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13. ABSTRACT (Maximum 200 Words)

We developed and tested a novel enzyme/prodrug strategy for cancer gene therapy using Carboxypeptidase A (CPA) to enzymatically convert the prodrug methotrexate-α-phenylalanine (MTX-Phe) to methotrexate (MTX). CPA is normally synthesized as a zymogen that requires proteolytic removal of its pro-peptide by trypsin in order to gain catalytic activity. In **Specific Aim I** in order to use CPA as a therapeutic gene we engineered a battery of mutant forms of CPA which were designed to be activated by normal cellular secretory processes. We found that the best mutant, termed CPA_{ST3}, was secreted from tumor cells in an active form and biochemical analysis revealed that it had similar activity and substrate specificity to the wild-type enzyme. In **Specific Aim II** we produced recombinant retroviruses coding for this mutant form of CPA and demonstrated that tumor cells infected with these viruses were potently sensitized to MTX-Phe and that there was a significant "bystander effect" even when less than 10% of the tumor cells were expressing CPA. We were unable to carry-out *in vivo* analysis of the MTX-Phe based system due to the unexpected instability of MTX-Phe *in vivo*. However, we did synthesize an active-site mutant of CPA_{ST3} to convert the stable prodrug MTX-3-cyclopentyl-tyrosine.

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FOREWORD

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Introduction:

The treatment of patients with advanced breast cancer using chemotherapeutic agents is often limited due to the inherently toxic nature of these drugs to rapidly dividing tissues (1). Tumor specific conversion of an inactive prodrug to a chemotherapeutic agent can overcome this limitation (2). Therefore, we proposed to develop and test a novel enzyme/prodrug strategy for cancer gene therapy. We chose Carboxypeptidase A (CPA) as the therapeutic gene based on its ability to enzymatically convert the prodrug methotrexate-α-phenylalanine (MTX-Phe) to the toxic chemotherapeutic agent methotrexate (MTX) (3-4). By targeting expression of CPA to tumor cells it is expected that systemically administered MTX-Phe will be converted to high intra-tumoral concentrations of MTX. This system will lead to heightened tumor cell kill without the associated systemic toxicities. CPA is normally synthesized as a zymogen that requires proteolytic removal of its pro-peptide by trypsin in order to gain catalytic activity (5). In order to use CPA as a therapeutic gene we engineered mutant forms of CPA that are activated through normal cellular secretory processes (6).

Specific aim1: Design and Characterization of (Carboxypeptidase A) CPA molecules which are efficiently processed by prohormone convertases within the secretory pathway.

We have now completed this aim and the publications in *Human Gene Therapy* and *Cancer Research* summarize these results (included as Appendices I and II).

The gene therapy strategy described depends upon the unique ability of the pancreatic protease Carboxypeptidase A (CPA) to remove a blocking amino acid from the prodrug Methotrexate-α-phenylalanine (MTX-Phe) thus converting it to the chemotherapy agent Methotrexate (MTX). This system has previously been described utilizing antibody dependent enzyme prodrug therapy (ADEPT) where the CPA was activated through trypsin dependent zymogen cleavage of its inhibitory propeptide (3-4). We endeavored to develop a gene dependent enzyme prodrug therapy (GDEPT) utilizing the same enzyme/prodrug combination. However, this required development of mutant forms of CPA which could be enzymatically activated in the absence of trypsin dependent zymogen cleavage (5).

Briefly, the original proposal described limited mutations in the propeptide of CPA in order to facilitate the endogenous activation by the PACE/Furin family of propeptidases; since these enzymes are ubiquitously expressed and should be found in all cell and tumor types (6). However, despite activation in the presence of over-expression of PACE/Furin family of enzymes the original CPA mutant was not activated by endogenous levels of prohormone convertases. Therefore, a battery of PACE cleavage site mutants were synthesized in order to simulate the multi-step nature of trypsin dependent activation that has been described for CPA. For details of the particular mutants see Appendix I figure 1 and Appendix II figure 1.

From this battery of mutants the CPA_{ST3} molecule was selected as the best mutant based on the ability of it to be fully processed to the mature form by endogenous proteases after transient transfection of cells in culture (Appendix II figure 2) or after retroviral transduction of tumor cells (Appendix II figure 3). The specificity of this PACE/Furin dependent activation was further elucidated by co-transfecting a specific inhibitor of PACE/Furin which resulted in a ~50% inhibition of CPA activation (Appendix II figure 2). Further, peptide sequencing of the liberated amino-terminus revealed that endogenous activation of CPA_{ST3} occurred at the predicted site. Finally, in order to more carefully evaluate the catalytic activity of these endogenously activated mutants they were purified by affinity chromatography and their catalytic activity assayed by spectrophotometric monitoring of a synthetic substrate. These studies revealed that CPA_{ST3} had a similar Km, Vmax, and specific activity when compared to the trypsin activated wild-type enzyme (Appendix II, Table I).

Specific aim2: *In vitro* characterization of the CPA/MTX-Phe, enzyme/prodrug system.

The majority of the *in-vitro* characterization of CPA_{ST3} has now been completed. This was published in *Cancer Research* in 2000 (see Appendix II).

The major thrust of these studies was that retrovirally infected cells that express CPA become sensitized to MTX-Phe in a time and dose dependent manner (See Appendix II figures 3, 4, and 5). Obviously as controls, non CPA-expressing cells were resistant to the prodrug even at concentrations up to a 1000-fold higher (See Appendix II, Table II). This has been confirmed in various tumor cell-lines from different tissue types (breast, head and neck, as well as liver) (data not shown).

In the initial application we had also proposed that we would construct a CPA molecule that would remain cell surface associated. Since wild type CPA is secreted, there is a possibility that it may enter the circulation and convert MTX-prodrugs to MTX at a non-tumor site. This would defeat the whole point of enzyme prodrug therapy, (*i.e.* tumor specific conversion of a non-toxic prodrug to a toxic drug thereby sparing normal tissue from high concentrations of the drug.) We have been successful in constructing a form of CPA_{ST3} that remains attached to the cell surface as an active enzyme. This was accomplished by fusing the DAF (decay accelerating factor) C-terminal membrane insertion and phosphoinositol linkage signals onto the C-terminus of CPA (Appendix II figure 6a and reference 7). Expression of this fusion protein results in cell associated CPA, as detected by western blot, without secretion of the enzyme into the media (Appendix II figure 6b). In addition, by flow-cytometry we further demonstrated that this CPA_{DAF} fusion protein is also cell-surface accessible (Appendix II figure 6c). Finally, growth inhibition assays revealed that the soluble and cell surface enzymes could both sensitize tumor cells to MTX prodrugs, and that they had similar substrate specificities to each other and to that which had previously been published for CPA (Appendix II, Table III and reference 8).

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To more fully evaluate the utility of both the soluble and secreted forms of endogenously activated CPA for GDEPT we performed traditional colony formation assays using different titers of retroviral supernatants (Appendix II figure 7), and we also developed a novel co-culture assay for bystander killing (Appendix II figure 8). These experiments revealed that both the soluble and cell-surface associated forms of CPA were able to sensitize tumor cells to MTX-Phe when less than 10% of the cells were infected with retroviruses coding for the enzymes of interest. However, when both expressing and non-expressing cells were co-cultured in a two-chambered tissue culture well, where a semi-permeable membrane separated the two populations of cells, the advantage of the cell-surface enzyme become more obvious. Under these circumstances cells non-transduced and located in the adjacent well were significantly more sensitized to MTX-Phe by the soluble enzyme than by the cell-surface form of CPA. This is likely due to diffusion of the soluble, secreted, and active enzyme away from the site of transduction and catalytically converting the MTX prodrugs to MTX at a site distant from the transduced cells. This series of experiments may at least in part show the benefit of a cell-surface enzyme for GDEPT therapy. By expressing the enzyme on the surface of the cell it enabled a potent bystander effect even when very few (<10%) of the cells were expressing the enzyme when sensitization was occurring in close proximity to the transduced cells. However, by localizing the enzyme and not allowing it to diffuse from the site of transduction it also prohibited non-specific prodrug activation (*i.e.* at a site distant from the tumor) which was not the case for the soluble enzyme.

SPECIFIC AIM 3: *In vivo* evaluation of the CPA/MTX-Phe enzyme/prodrug system.

To complete this aim we developed a number of reagents. We have constructed adenoviral gene delivery vectors that can be used to deliver the CPA_{ST3} and the CPA_{DAF} genes to *in vivo* tumors. These have been tested *in vitro* and expression of the appropriate polypeptides has been observed (data not shown). However, we were not able to perform the in vivo analysis due to the unexpected report that MTX-Phe, unlike previous predictions, is not stable in vivo; rather, it is rapidly converted to MTX (9). Fortunately, an advancement of the CPA / MTX prodrug ADEPT strategy was reported where new more stable prodrugs were synthesized and active-site mutants of CPA were engineered that were able to cleave these second generation prodrugs (9). Therefore, we are in the process of developing active site mutants of CPA that we will use to further evaluate this enzyme/prodrug strategy.

KEY RESEARCH ACCOMPLISHMENTS:

• Design, synthesis, and characterization of endogenously activated forms of CPA for use in GDEPT.

• Endogenously activated CPA_{ST3} is fully activated on secretion from a number of different tumor cell types.

• CPA_{ST3} is structurally and functionally identical to trypsin activated wild-type CPA

• Design, synthesis, and characterization of cell-surface localized forms of CPA for use in GDEPT.

• CPA_{DAF} is activated by endogenous intracellular propeptidases and remains attached to the cell-surface through a phosphotidyl inositol linkage.

• Development and characterization of both retroviral and adenoviral constructs expressing endogenously activated soluble and cell-surface CPA.

• Demonstration of a 1000-fold sensitization to MTX prodrugs following viral transduction of cells in culture with recombinant retroviruses coding for soluble or cell-surface localized forms of CPA.

• Cell-surface localization of CPA_{DAF} provides a potent bystander effect by sensitizing non-transduced cells to MTX prodrugs, but also has decreased diffusion of active enzyme away from the site of transduction.

• Although not part of the original proposal while funded on this grant I also performed a number of experiments to evaluate an alternative enzyme/prodrug therapy based upon both the bacterial and yeast cytosine deaminase genes and the prodrug 5-flucytosine. The studies on this system are described in Appendices III and IV.

REPORTABLE OUTCOMES:

PAPERS PUBLISHED (Attached as Appendices I - IV)

Hamstra, D.A. and Rehemtulla, A. (1999) Toward an Enzyme/Prodrug Strategy for Cancer Gene Therapy: Endogenous activation of Carboxypeptidase A mutants by the PACE/Furin family of propeptidases. *Human Gene Ther*, 10:235-248.

Hamstra, D.A., Pagé, M., Maybaum, J., and Rehemtulla, A. (2000) Expression of Endogenously Activated Secreted or Cell-Surface Carboxypeptidase A Sensitizes Tumor Cells to MTX-α-Peptide Prodrugs. *Cancer Res.* 60:657-665.

Hamstra, D.A., Rice, D.J., Pu, A., Oyedijo, D., Ross, B.D., and Rehemtulla, A. (1999) Combined Radiotherapy and Enzyme/Prodrug Therapy for Head and Neck Cancer in an Orthotopic Animal Model. *Rad Research.* 152:499-507.

Hamstra, D.A., Rice, D.J., Fahmy, S., Ross, B.D., and Rehemtulla, A (1999) Enzyme/Prodrug Therapy for Head and Neck Cancer Using a Catalytically Superior Cytosine Deaminase. *Human Gene Ther.* 10:1993-2003.

ABSTRACTS & PRESENTATIONS

Hamstra, D.A. and Rehemtulla, A. Poster Presentation. Carboxypeptidase A (CPA) mutants and methotrexate-α-peptide prodrugs for virus dependent enzyme prodrug therapy (VDEPT). "Annual Meeting of the American Association of Cancer Research." March 28 - April 1, 1998. New Orleans, LA.

Hamstra, D.A. and Rehemtulla, A. Oral Presentation. Expression of Endogenously Activated Soluble or Cell-Surface Carboxypeptidase A Sensitizes Tumor Cells to Methotrexate Prodrugs. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

Hamstra, D.A., Rice, D.J., Oyedijo, D., Ross, B.D., and Rehemtulla, A. Oral Presentation. Yeast Cytosine Deaminase is Functionally Superior to E. Coli Cytosine Deaminase in Enzyme Prodrug Cancer Gene Therapy *In Vitro* and *In Vivo*. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

Rice, D.J., **Hamstra, D.A.**, Pu, A., Oyedijo, D., Ross, B.D., and Rehemtulla, A. Poster Presentation. Bacterial Cytosine Deaminase Gene Therapy and Radiotherapy Inhibit Tumor Growth and Enhance Survival in an *In Vivo* Model of Head and Neck Cancer. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

AWARDS

1999	Daniel A. Hamstra
	Young Investigator Award. The American Society of Gene Therapy
1998	Daniel A. Hamstra
	Thomas Baum Award. The University of Michigan Department of Pharmacology

DOCTORAL DEGREE

2001   Daniel A. Hamstra
    Ph.D. Pharmacology. The University of Michigan Medical School
    Thesis: "Development of novel gene dependent enzyme
    prodrug therapies for the treatment of head and neck cancer."
    Co-Mentors: Alnawaz Rehemtulla, Ph.D and
    Jonathan Maybaum, Ph.D

EMPLOYMENT

2/1/01 - 6/30/01	Postdoctoral Research Scientist. The Department of Radiation Oncology. The University of Michigan Medical Center.
7/1/01 - 6/30/02	Internship. Internal Medicine. The University of Michigan Medical Center.
7/1/02 - 6/30/06	Residency. Radiation Oncology. The University of Michigan Medical Center.

CONCLUSION

Based upon the published results using CPA in ADEPT studies for cancer therapy (3-4, 8-9) we setout to develop a GDEPT system using endogenously activated forms of CPA. Our initial goal was to by-pass the trypsin dependent zymogen activation of CPA (5) in order to generate mature extra-cellular CPA to cleave the administered prodrugs. The studies reported in Appendices I and II support the conclusion that we fully realized this goal. CPA_{ST3} is processed to a mature form in a number of different cells lines by endogenous levels of the prohormone convertases (PC). The fact that all seven members of the PC family could activate these CPA mutants makes it very unlikely that any tumor tissues could be identified which were resistant to activation (6). The endogenous activation of CPA by intracellular prohormone convertases is attractive because the enzyme produced appears to be structurally and functionally identical to the native enzyme, and thus all of the results obtained using ADEPT based studies should be readily extended to this new system. In addition, other members of the pancreatic carboxypeptidase family have a similar zymogen activation pathway; and therefore, they too could be adapted for GDEPT using PC based activation (5). Since the other mammalian pancreatic carboxypeptidases have different substrate specificities they have also been proposed for ADEPT, and these different substrate specificities could be capitalized on in order to activate prodrugs with different blocking groups (10).

In addition to being the first report of MTX in a GDEPT this is also one of the first studies to utilize both an extra-cellular enzyme and an extra-cellularly generated metabolite. This extra-cellular production resulted in a substantial bystander effect by both limiting the toxic accumulation within the transduced or "factory" cells while also providing rapid dissemination of the drug to neighboring tissues through extra-cellular diffusion. The generation of active MTX in the extra-cellular environment resulted in a significant bystander effect even with very low transduction efficiencies.

The utilization of a cell-surface localized form of an enzyme for GDEPT is also an area of study that could be expanded to other GDEPT systems. One previous report, using bacterial carboxypeptidase G2 (CPG2) and mustard based prodrugs, utilized a cell-surface localized enzyme due to the poor uptake of the prodrug into the cells; and therefore, a low rate of activation of the prodrug by an intra-cellularly expressed enzyme (11). These authors demonstrated a significant improvement in the cleavage of the prodrug and of the therapeutic response using CPG2 fused to the transmembrane domain of a receptor protein tyrosine kinase as compared to the intracellular expression of the same enzyme. However, the use of a protein-based linkage may not be feasible for enzymes that require conformational freedom in order to assume the appropriate protein-fold for activity. When we constructed fusion proteins between the transmembrane domain of human tissue-factor and either CPA or cytosine deaminase we were unable to measure any enzymatic activity for either enzyme despite the production of an appropriately sized fusion protein that was membrane localized (D.A.H. and A.R., unpublished data). In contrast, the utilization of a lipid linkage appears to have allowed for an active cell-surface enzyme that maintains the activity and substrate specificity of the native soluble enzyme.

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The CPA-based GDEPT is also unique in that the inactivation of MTX to the prodrug form is not due to de-toxification of the drug itself, for MTX-α-peptides can still effectively bind to and inhibit DHFR (3). Rather, the decrease in toxicity occurs by sequestering the prodrugs from their target enzyme through the inhibition of their active cellular uptake. This phenomena need not be limited to MTX, for other chemotherapeutics that enter the cell through either active transport or even by passive diffusion could also theoretically by conjugated to lipophobic blocking groups and thus inhibit their cellular uptake. Significant work has recently been performed to elucidate the substrate specificity of the carboxypeptidases (9), and these proteases, therefore, could most likely be engineered to activate other classes of prodrugs. The first-step toward the realization of this strategy has been achieved through the development and report of a number of different prodrugs of folate-based TS-inhibitors which are substrates for CPA (12-13). In addition to these reported compounds, there are also literally dozens of other chemotherapeutics either in investigation or on the market that could be used in this GDEPT strategy. Many of the enzymes involved in the metabolism of folate have been targeted by either specific or broad-based anti-folate therapy using drugs that require active cellular uptake through the reduced folate transporter(14), and which could therefore likely be readily converted to α-peptide prodrugs. Many of these compounds have increased cytotoxicity relative to MTX and are still active even against MTX-resistance cell-lines. For example, in order to alleviate tumor-based resistance due to decreased polyglutamation of MTX, agents have been developed that do not require the action of folylpolyglutamate transferase for potent cytotoxicity. Therefore, they exhibit potent inhibition of DHFR and cytotoxicity even toward head and neck cell-lines that are resistant to MTX due to deficient polyglutamation (15-16). Thus, just as combination regimens have been developed using traditional chemotherapy; one could envision the development of multiple chemotherapeutic prodrugs which are all activated by a CPA based GDEPT and which synergistically interact to increase cytotoxicity and limit the development of resistant tumor populations. Unlike the recent reports of fusion proteins with multiple enzymatic activities (17) or the use of bi-cistronic viral vectors (18) the strategy envisioned here would allow for the activation of multiple prodrugs through the action of one enzyme.

Finally, although significant work has been done to evaluate this GDEPT system through *in vitro* analysis the study of the system using animal models of breast cancer have yet to be undertaken. The orginal combination of CPA and MTX-Phe has proven not to be workable *in vivo*. However, further studies using stable MTX prodrugs and an altered substrate specificity form of CPA may yet prove the utility of this GDEPT in the treatment of breast cancer.

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Toward an Enzyme/Prodrug Strategy for Cancer Gene Therapy: Endogenous Activation of Carboxypeptidase A Mutants by the PACE/Furin Family of Propeptidases

DANIEL A. HAMSTRA[1,2] and ALNAWAZ REHEMTULLA[1]

ABSTRACT

In an effort to develop a gene-dependent enzyme/prodrug therapy (GDEPT) for tumor-specific delivery of methotrexate (MTX) we have chosen to construct mutant forms of carboxypeptidase A1 (CPA) that circumvent the requirement for trypsin-dependent activation. The basis of this strategy is that methotrexate-α-peptides are inefficient substrates for the reduced folate carrier (RFC) and hence cannot be internalized by cells. However, the blocking amino acid can be cleaved by CPA to liberate MTX, which is then internalized by the RFC, resulting in inhibition of dihydrofolate reductase and cytotoxicity. A battery of mutant CPAs was generated, in which the putative trypsin cleavage sites in the propeptide were mutated to the consensus recognition sequence for mammalian subtilisin-like propeptidases. These mutant forms of CPA were evaluated for expression, activation, and catalytic activity by transiently transfecting them into COS-1 cells both in the absence and in the presence of cotransfected propeptidases. CPA$_{95}$ was identified as the most efficiently cleaved mutant, and further studies of this mutant indicated that the endogenously activated enzyme had kinetic parameters identical to those of the trypsin-activated wild-type protein. In addition, endogenously activated CPA$_{95}$ could effectively sensitize cells to MTX-Phe in culture, decreasing the IC$_{50}$ of MTX-Phe from 25- to 250-fold in squamous cell carcinoma cells expressing active CPA as compared with the parental lines.

OVERVIEW SUMMARY

To utilize CPA in an enzyme/prodrug strategy for cancer gene therapy it was necessary to develop mutant forms of CPA that do not require trypsin-dependent zymogen activation. A battery of CPA mutants was developed and screened for their expression and ability to be activated by the subtilisin-like prohormone convertases. CPA$_{95}$, the mutant that exhibited the best secretion, activation, and catalytic activity, was then purified and enzymatically characterized, demonstrating that it had a kinetic profile identical to that of trypsin-activated wild-type CPA. Finally, the utility of this enzyme/prodrug system was evaluated in an *in vitro* assay.

INTRODUCTION

A MAJOR OBSTACLE to the clinical treatment of cancer using chemotherapy is the lack of antitumor selectivity exhibited by most chemotherapeutic agents (Krakoff, 1987). Enzyme/prodrug approaches have been used as a means to overcome the lack of selectivity of chemotherapeutic drugs for neoplastic cells over normal cells (Culver *et al.,* 1992; Huber *et al.,* 1994; Chen and Waxman, 1995; Marais *et al.,* 1996). The principle of these systems is that targeting of an activating enzyme to tumor cells results in the conversion of a systemically administered nontoxic prodrug to high intratumoral concentrations of an anticancer drug. A number of gene-based approaches have been developed as enzyme/prodrug therapies, and these have been collectively labeled gene dependent enzyme/prodrug therapy (GDEPT) (for review see Connors, 1995). The prototype of a gene-based enzyme/prodrug system is the herpes simplex virus thymidine kinase (HSV TK) in conjunction with the antiviral drug gancyclovir (GCV), which is preferentially phosphorylated by the HSV TK and subsequently by cellular kinases to GCV triphosphate, which disrupts DNA synthesis (Culver *et al.,* 1992). This system has undergone widespread testing *in vitro*, in preclinical studies, and in clinical trials (Culver *et al.,* 1992; Moolten, 1986; Moolten and Wells, 1990; Huber *et al.,* 1991).

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Methotrexate (MTX) is a good choice for an enzyme/prodrug strategy in that it is one of the most widely used chemotherapeutics in the treatment of solid tumors, and its pharmacodynamics and toxicities have been well established (Ensminger, 1984; Tattersall, 1984). The toxicity of MTX predominantly occurs in the gastrointestinal tract and the bone marrow; therefore, the local production of MTX within a tumor distant from these sites should allow for high-dose chemotherapy without the normal dose-limiting toxicities. Previous work has established that MTX-α-peptides (Fig. 1) are relatively nontoxic prodrug forms of MTX owing to their inability to be internalized by the reduced folate transport system (Vitols *et al.*, 1995; Perron and Page, 1996; Smith *et al.*, 1997). However, these MTX-α-peptide prodrugs can be enzymatically converted to MTX by the enzyme carboxypeptidase A (CPA) *in situ*.

The utility of the CPA/MTX-α-peptide system has been studied using an antibody-dependent enzyme/prodrug therapy (ADEPT) model by first administering a tumor-specific antibody conjugated to purified trypsin-activated CPA (Vitols *et al.*, 1995; Perron and Page, 1996; Smith *et al.*, 1997) and subsequently administering the MTX prodrug. These reports demonstrated the efficacy of this system *in vitro*; one report describing novel MTX-α-peptides in conjunction with the T268G mutant of human CPA1 demonstrated that these prodrugs were 800-fold less cytotoxic than MTX in the absence of the antibody:CPA conjugate, but they had cytotoxicity similar to that of MTX in the presence of the antibody:CPA conjugate (Smith *et al.*, 1997). However, ADEPT systems are plagued by a number of problems including cost and difficulties with development and purification of antibodies, immunogenicity of antibodies, accessibility of tumor to the enzyme/antibody conjugate, and background conversion of prodrugs owing to localization of antibody conjugates to inappropriate tissues (Deonarain *et al.*, 1995).

We therefore hypothesized that a gene-based enzyme/prodrug strategy might take advantage of the CPA/MTX-α-peptide system while avoiding the inherent difficulties imparted by an antibody derived approach. Carboxypeptidase A is a zinc metalloprotease normally synthesized and secreted by the exocrine pancreas (Oppezzo *et al.*, 1994). CPA is a zymogen that requires proteolytic removal of its propeptide by trypsin within the lumen of the small intestine in order to gain catalytic activity. Since trypsin is localized to the small intestine there would be no proteolytic activation of CPA expressed in tumor cells. Therefore, to be useful in GDEPT it is necessary to generate a CPA mutant that is expressed as an active enzyme in a

trypsin-independent manner. Although the gene for CPA has been cloned (Quinto *et al.*, 1982) and expressed in yeast (Gardell *et al.*, 1985), its expression in mammalian cells has not been reported. The propeptide of the mammalian pancreatic CPAs, which is removed by trypsin cleavage, has been shown to bind tightly to the catalytic domain of CPA and to act as a potent inhibitor of the enzyme (Vendrell *et al.*, 1990; Segundo *et al.*, 1982). Efforts by other laboratories to express the mature protein lacking the propeptide have been unsuccessful because the propeptide is required for proper folding and secretion of the enzyme (Phillips and Rutter, 1996).

The goal of the work outlined here was to develop a system whereby carboxypeptidase A could be secreted from cells as an active enzyme owing to removal of the prosequence by endogenous cellular propeptidases. Paired basic amino acid-claving enzyme (PACE), or furin, is a Ca^{2+}-dependent serine protease that processes proteins in the constitutive secretory pathway (Rehemtulla and Kaufman, 1992a). PACE is the prototypical member of the subtilisin-like proprotein convertases, which include the yeast homologue Kex2 (EC 3.4.21.61) and the mammalian propeptidases PC1/3, PC2, PC4, PC5/6, PACE4 (reviewed in Rehemtulla and Kaufman, 1992a), and PC7/PC8 or lymphoma proprotein convertase (Bruzzaniti *et al.*, 1996; Constam *et al.*, 1996; Meerabux *et al.*, 1996; Seidah *et al.*, 1996). These proteases are involved in the maturation of proproteins by cleaving after dibasic or tetrabasic sites in order to remove their propeptides (Rehemtulla and Kaufman, 1992b). We report here the mutation of the propeptide of pro-CPA such that it is rendered a substrate for the subtilisin-like proprotein convertases, and we propose an enzyme/prodrug strategy whereby the gene for a mutant form of CPA would be delivered to tumor cells, enabling them to synthesize, proteolytically activate, and secrete active CPA in to the tumor milieu. Tumor-specific synthesis of active CPA should lead to intratumoral conversion of MTX-Phe to MTX, resulting in high intratumoral concentrations of MTX without systemic toxicity.

MATERIALS AND METHODS

Rat Carboxypeptidase A1

Except where indicated otherwise all common molecular biological techniques were performed according to Sambrook *et al.* (1989). The cDNA for rat pro-CPA1 was isolated from a rat pancreatic cDNA library (kindly provided by C. Dickenson, De-
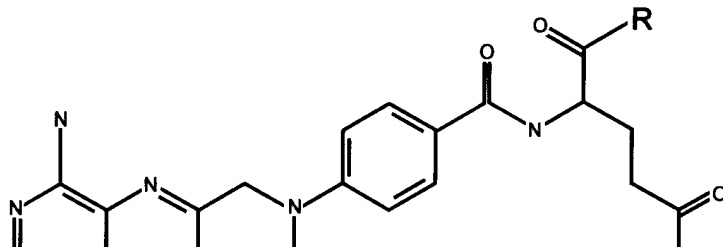

FIG. 1. Structure of MTX-α-R prodrugs. All R groups are L-amino acids attached to the α-carbonyl of MTX via a standard peptide bond.

partment of Pediatrics, The University of Michigan, Ann Arbor), using polymerase chain reaction (PCR) primers that introduce a SalI site 5' to the endogenous Kozak sequence and start codon and an XbaI site 3' to the stop codon (see below). PCR was performed using Expand high-fidelity polymerase (Boehringer GmbH, Mannheim, Germany), and the PCR product obtained was cloned into the expression vector pZ, a PUC 18-based vector that drives protein expression from the adenovirus major late promoter and contains an internal ribosomal entry site allowing for expression of the neomycin resistance gene (neo^R) off from one bicistronic mRNA; in addition, a simian virus 40 (SV40) origin of replication allows for high levels of protein production in COS-1 cells (kindly provided by the Genetics Institute, Boston, MA).

Mutagenesis

A four-primer PCR-based mutagenesis strategy (Huguchi, 1990) was used to introduce all of the mutations that were subsequently verified by DNA sequencing using Sequenase 2.0 (U.S. Biochemical, Cleveland, OH) or by the University of Michigan DNA Sequencing Core. For consistency all numbering is according to the rCPA1 cDNA. The CPA_{95} mutant was constructed using 5' RCPA and 3' RCPA as the flaking primers and 5' $RCPA_{95}$ and 3' $RCPA_{95}$ as the mutagenic primers. The CPA_{54} and CPA_{85} mutants were made in an analogous fashion using 5' and 3' $RCPA_{54}$ or $RCPA_{85}$ primers, respectively. Double mutants ($CPA_{54/95}$ and $CPA_{85/95}$) were constructed by using CPA_{95} as the template DNA. The oligonucleotides (oligos) used were as follows:

5' RCPA: GCTCTAGAGTCGACCCTTCTCACCAT-
              GAAGAGAC
3' RCPA: GCTCTAGAGAATTCGGTGGGGGATAA-
              GGGCTGCTT
5' $RCPA_{95}$:   CGCCAGAAAAGGGCCCTGTCCACT-
              GACTCTTTCAATTATG
3' $RCPA_{95}$:   GGGCCCTTTTCTGGCGGGCAGACATCT-
              GCTGTTTC
5' $RCPA_{54}$:   GGACTTCTGGCGGGACCGTGCCCG-
              GCGCGGGATCCCCATTGATGTCAGAG
3' $RCPA_{54}$:   CTCTGACATCAATGGGGATCCCGCGC-
              CGGGCACGGTCCCGCCAGAAGTCC
5' $RCPA_{85}$:   GAAAGCATTCTTGGAATATCGCCGGC-
              GAAGATATGAGATCATGATTGAAGATG
3' $RCPA_{85}$:   CATCTTCAATCATGATCTCATATCTTCGC-
              CGGCGATATTCCAAGAATGCTTTC

Prohormone convertase expression plasmids

Plasmid expression vectors for PACE/furin, PACE4, and Kex2 have all been previously described (Rehemtulla and Kaufman, 1992b). Rat PC7 (a gift from N. Seidah, IRMC, Montreal, Quebec, Canada) was subcloned into the expression vector pZ.

Transfections

COS-1 African green monkey kidney cells were cultured and transfected using the DEAE-dextran procedure as previously described (Rehemtulla and Kaufman, 1992b). To harvest CPA for purification or for enzyme assays 48 hr posttransfection

plates were washed three times with phosphate-buffered saline (PBS) and then incubated in serum-free medium (Optimem; GIBCO-BRL, Gaithersburg, MD) for an additional 24 hr, at which time the supernatants were harvested, nonadherent cells were spun down by centrifugation for 15 min at $1000 \times g$, and the conditioned medium frozen at $-70°C$ for subsequent analysis. For experiments requiring metabolic labeling of proteins, [^{35}S]Met/Cys (Pro-mix; Amersham, Arlington Heights, IL) was used according to the protocols previously described (Rehemtulla and Kaufman, 1992b).

Western blotting

The expression of PACE/furin was monitored by Western blotting as previously described (Rehemtulla and Kaufman, 1992b), using a polyclonal anti-PACE antibody followed by enhanced chemiluminescence (Pierce, Rockford, IL). The expression of both intracellular and extracellular CPA or CPA mutants was monitored using a rabbit polyclonal anti-bovine CPA antiserum (Cemicon, Temecula, CA) followed by enhanced chemiluminescence.

Trypsinization of CPA

Trypsinization of pro-CPA and pro-CPA mutants was performed using 250 ng of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO) per milliliter of conditioned medium from transiently transfected COS-1 cells. Trypsinization was allowed to occur at 37°C for 3 hr and stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to 0.1 mM.

Enzyme activity assays

CPA activity was measured using a spectrophotometric assay for cleavage of the synthetic substrate N-(3-[2-furyl]acryoyl)-Phe-Phe (FAPP [Sigma]; Peterson et al., 1982) as previously described. For kinetic analysis FAPP hydrolysis was measured at 330 nm and 25°C in the presence of 2.0×10^{-10} mol of enzyme while the substrate concentration was varied from 1×10^{-5} to 5×10^{-4} M. Enzyme kinetic rates were determined from initial velocities using $\epsilon = 2000$ M^{-1}. A coupled spectrophotometric assay for MTX-Phe cleavage in the presence of carboxypeptidase G_2 was performed as previously described (Kuefner et al., 1989). Data were plotted and analyzed using the nonlinear regression analysis function in the Graphpad Prism (Graphpad Software, San Jose, CA) software package. The specific activity of purified rCPA was determined by comparison with bovine CPA (Sigma).

Purification of recombinant RCPA

Carboxypeptidase potato inhibitor (CPI) affinity chromatography was used for the isolation of mature CPA (Ager and Hass, 1977). Twenty-five milligrams of purified CPI (Sigma) was conjugated to 1 ml of Affi-Gel 10 Affinity Matrix (Bio-Rad, Hercules, CA) according to the manufacturer instructions. Conditioned media containing recombinant CPA or CPA_{95} in the presence of 0.1 mM PMSF either with or without pretrypsinization were dialyzed under vacuum against 4 liters of the binding buffer (250 mM NaCl, 20 mM Tris-HCl [pH 8.0]) using a 10-kDa cutoff dialysis membrane. The concentrated dialysate

was then loaded onto the CPI column preequilibrated with the binding buffer. After loading the column was washed with 10 bed volumes of the binding buffer, and the CPA was eluted at high salt and pH (1 M NaCl, 0.1 M NaHCO$_3$ [pH 11.4]). Fractions were analyzed by sodium dodecyl sulfate-polyarylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Those fractions determined to contain purified CPA were pooled, adjusted to pH 8.0, their concentration determined by the Lowry protein assay (Bio-Rad) using bovine serum albumin as a standard, and then used in kinetic assays.

Cytotoxicity assay

SCCVII cells, a mouse squamous cell carcinoma line (American Type Culture Collection [ATCC], Rockville, MD), and UMSCC6, a human squamous cell carcinoma cell line (Krause et al., 1981), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with pZ.CPA$_{95}$ alone or in combination with pMT3.PACE.sol and stable clones selected on the basis of neomycin resistance. The highest expressing clones were isolated on the basis of protein expression and sensitivity to MTX-Phe. Cytotoxicity assays were performed using the Sulforhodamine B (SRB) assay (Skehan et al., 1990). Cells were plated at a density of 3000 cells/cm^2 in a 96-well plate; 12–18 hr after plating the medium was changed to that supplemented with vehicle (PBS), MTX, or MTX-α-phenylalanine (MTX-Phe; Molecular Probes, Eugene, OR). The cells were left cultured with the drug for 72 hr, at which point they were fixed and stained according to Skehan et al. Data plotted represent the mean and standard error of eight replicate wells.

RESULTS

Cloning and mutagenesis of rCPA1

To evaluate the use of rat CPA1 in an enzyme/prodrug strategy for cancer gene therapy we set about the cloning and expression of rat pro-CPA1 in a mammalian cell culture system. To clone the cDNA for rat pro-CPA1, PCR oligos were designed on the basis of the published sequence of rat CPA1 (Quinto et al., 1982) (see Materials and Methods). The polymerase chain reaction was performed using a rat pancreatic cDNA library as template, resulting in a single product of the expected size (~1.3 kb), which was cloned into the mammalian expression vector pZ. The nucleotide sequence of the isolated cDNA was identical to the published sequence for rat pro-CPA1.

The penultimate trypsin cleavage site in rCPA at Arg-95 (FQAR$_{95}$ ↓) (Oppezzo et al., 1994) was mutated to create a consensus recognition sequence for PACE (**RQKR**$_{95}$ ↓) (Rehemtulla and Kaufman, 1992b), yielding the CPA$_{95}$ mutant (see Fig. 2). In addition, since trypsin has been shown to cleave pro-CPA in a number of other distinct sites within the propeptide, further consensus PACE cleavage sequences were introduced at these sites as well (see Fig. 2) (Vendrell et al., 1990). The second mutant, termed CPA$_{54}$, containing a PACE cleavage site at amino acid 54, was constructed by the mutation of the wild-type sequence (RDAARA$_{54}$) to contain a PACE cleavage site (**RARARR**$_{54}$ ↓). A third mutant, termed CPA$_{85}$, containing a PACE cleavage site at amino acid 85, was constructed by the mutation of the wild-type sequence (HGIS$_{85}$) to contain a PACE cleavage site (**RRRR**$_{85}$ ↓). In addition, double mutants were constructed, termed CPA$_{54/95}$ and CPA$_{85/95}$, by the introduction of the CPA$_{54}$ and CPA$_{85}$ mutations into CPA$_{95}$.

Expression of CPA constructs

To test the ability of the wild-type and mutant CPA constructs to produce pro-CPA in a mammalian expression system, the plasmids were transiently transfected into COS-1 cells. Forty-eight hours after transfection the cells were metabolically labeled with [^{35}S]Met/Cys for 5 min and the cell extracts were analyzed by SDS-PAGE followed by autoradiography (Fig. 3A). Cell extracts from all of the pro-CPA or pro-CPA mutant transfectants gave rise to a band at ~43 kDa. This band comigrated with β-actin; however, the increased intensity of the band when compared with mock-transfected cells indicates that all of these experssion plasmids yielded similar primary translation products. Further, this band at 43 kDa was specifically recognized by Western blot using a polyclonal anti-bovine CPA antibody (data not shown).
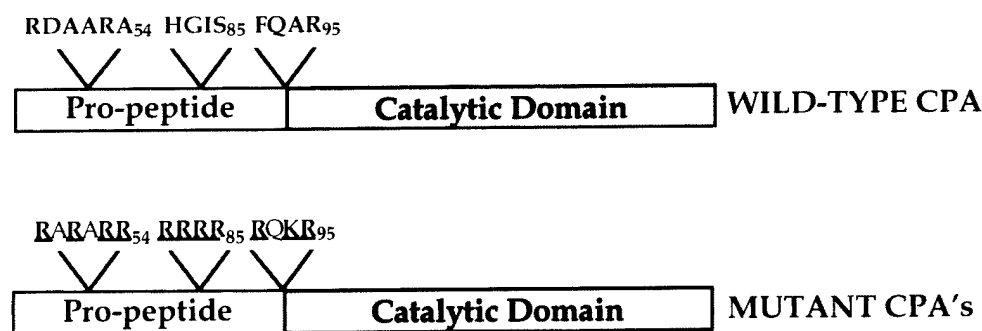

FIG. 2. Representation of the mutations introduced in the rCPA1 protein to facilitate subtilisin-like propeptidase cleavage. Wild-type CPA (top) and the various mutant CPAs (bottom) are depicted in diagrammatic form, with the amino acids shown for each mutated sequence. Residues representing the PACE cleavage site are indicated in boldface and underlined (**R**). Three of the mutants generated each contained one PACE cleavage site: CPA$_{54}$, CPA$_{85}$, CPA$_{95}$; and two mutants were generated with two PACE cleavage sites: CPA$_{54/95}$ and CPA$_{85/95}$.
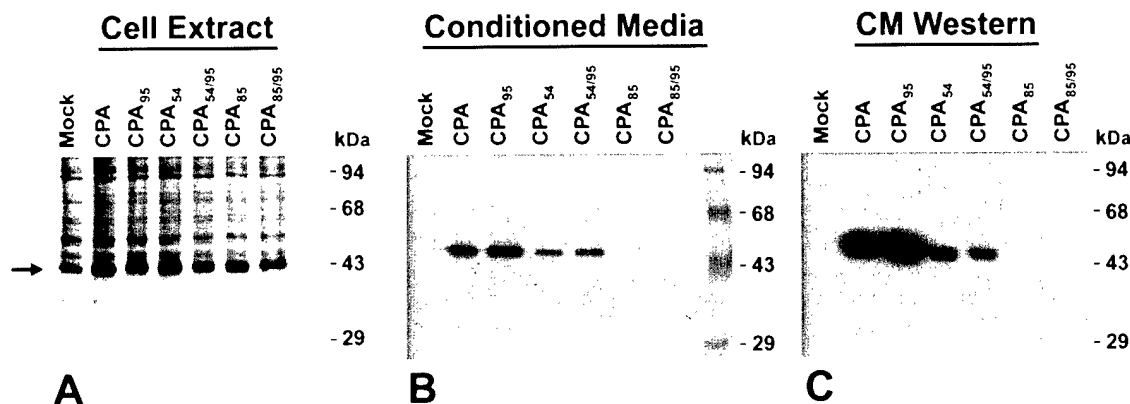
Cell Extract   Conditioned Media   CM Western

FIG. 3. Expression of wild-type and mutant CPAs in COS-1 cells results in a 43-kDa polypeptide. Expression plasmids for wild-type and mutant CPA constructs were transfected into COS-1 cells. Cells were labeled for 5 min with $[^{35}S]$Met/Cys, and cell extracts were analyzed by SDS-PAGE and autoradiography (**A**). In a separate experiment cells were labeled for 30 min and chased for 4 hr, at which time point the conditioned media were harvested and analyzed by SDS-PAGE and autoradiography (**B**) or by Western blot analysis using a CPA-specific polyclonal antibody (**C**). As control, COS-1 cells were transfected in the absence of DNA and analyzed as above (lanes labeled as Mock).

To examine the secretion of the wild-type and mutant CPA constructs, we performed a 4-hr chase following a 30-min pulse with $[^{35}S]$Met/Cys. The conditioned media were examined by SDS-PAGE and autoradiography (Fig. 3B) as well as Western blotting (Fig. 3C). Pro-CPA as well as all of the mutant pro-CPAs, except for pro-CPA$_{85}$ and pro-CPA$_{85/95}$, were secreted from the cells as witnessed by the appearance of a radiolabeled and immunoreactive band in the conditioned media with an apparent molecular mass of 43 kDa following a 4-hr chase. In contrast, pro-CPA$_{85}$ and pro-CPA$_{85/95}$ were not detected in the conditioned media by autoradiography of Western blot analysis (Fig. 3B and C). Expression of wild-type and mutant CPA constructs did not result in detectable cleavage of pro-CPA (43 kDa) to mature CPA (34 kDa). However, this was not unexpected for we have previously demonstrated that expression of proproteins in COS-1 cells results in poor processing (Rehemtulla and Kaufman, 1992b) owing to low levels of subtilisin-like propeptidases present in COS-1 cells and to the extremely high level of protein production from the transfected expression plasmid.

Reports have suggested that alterations in the proregion of rCPA can have detrimental effects on the folding, secretion, and catalytic activity of rCPA (Phillips and Rutter, 1996). Since it appeared that the secretion of pro-CPA$_{54}$ and pro-CPA$_{54/95}$ may have been less than that of the wild-type protein, we undertook pulse–chase experiments to assess in detail the secretion of wild-type CPA, pro-CPA$_{95}$, pro-CPA$_{54}$, and pro-CPA$_{54/95}$. Forty-eight hours posttransfection COS-1 cells were labeled with $[^{35}S]$Met/Cys for 30 min and then chased with media supplemented with excess cold Met/Cys (15 and 40 mg/ml). The cell extracts and conditioned media from duplicate wells were harvested at 0, 30, 60, 120, 180, and 240 min after a 30-min pulse and were analyzed by SDS-PAGE and autoradiography (Fig. 4). From these experiments it is apparent that both pro-CPA and pro-CPA$_{95}$ are rapidly and efficiently secreted from the cells as witnessed by their presence in the conditioned media at the earliest time point after labeling (30 min) and by the accumulation of protein in the conditioned media. In contrast, both the level and the rate of secretion of pro-CPA$_{54}$ and pro-CPA$_{54/95}$ were negatively impacted since the mutant proteins were not detected in the conditioned media until the 2-hr time point, and the absolute level of protein accumulation in the conditioned media was decreased when compared with pro-CPA and pro-CPA$_{95}$.

Processing of CPA mutants by subtilisin-like propeptidases

To test the ability of the wild-type and mutant proteins to act as PACE substrates the expression plasmids for wild-type CPA, CPA$_{95}$, CPA$_{54}$, and CPA$_{54/95}$ were cotransfected in COS-1 cells in the presence of a PACE expression plasmid. Forty-eight hours after transfection the cells were labeled with $[^{35}S]$Me/Cys for 30 min, and conditioned media were harvested following a 4-hr chase and analyzed by SDS-PAGE and autoradiography. Cells cotransfected with an expression plasmid for PACE along with the CPA expression plasmid secreted CPA predominantly in the 43-kDa proform (Fig. 5). In contrast, cells cotransfected with the PACE and CPA$_{95}$ expression plasmids secreted a 43-kDa band (pro-CPA) and a 34-kDa band (mature CPA) (Fig. 5). These apparent molecular weights are consistent with those published for the pro- and mature forms of rat CPA1 either purified from pancreas (Oppezzo *et al.*, 1994) or produced in yeast (Quinto *et al.*, 1982). In addition, coexpression of PACE and CPA$_{54/95}$ but not CPA$_{54}$ resulted in the appearance of this 34-kDa band.

Since pro-CPA$_{95}$ was both the most efficiently secreted and processed mutant we chose to characterize this protein further. To assess the ability of pro-CPA$_{95}$ to be activated by other members of the subtilisin-like serine protease family additional cotransfections were performed in COS-1 cells, using either pro-CPA or pro-CPA$_{95}$ along with expression plasmids for various subtilisin-like propeptidases: PACE, PACE4, PC7, or the yeast homolog Kex2. When the conditioned media from these co-
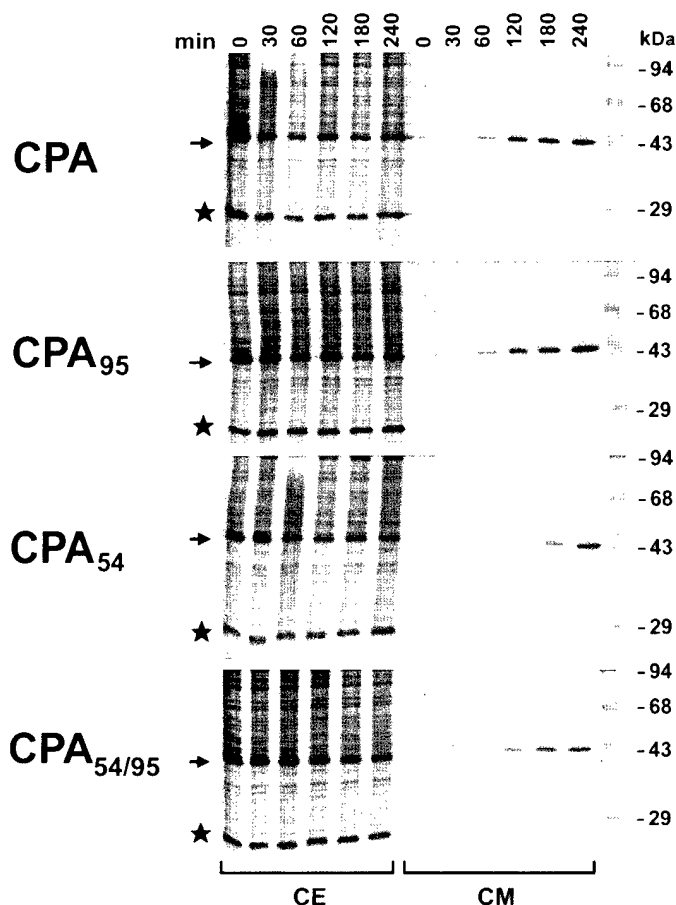
FIG. 4. Pulse–chase analysis of selected CPA mutants. The expression constructs for CPA, CPA_{95}, CPA_{54} and $CPA_{54/95}$ (from top to bottom) were transfected into COS-1 cells and metabolically labeled after 48 hr. Six duplicate dishes were transfected for each expression construct: one was harvested after a 30-min pulse (0 min) and the others were chased for 30, 60, 120, 180, and 240 min as indicated. Lanes labeled CE represent samples of cell extracts from each of the indicated time points. Lanes labeled CM represent conditioned media from the same dish at the indicated time points. The samples were resolved on 12% SDS-polyacrylamide gels followed by autoradiography. The 43-kDa pro-CPA band is indicated by arrows, and the neomycin gene product, which is contained on the same transcription cassette (bicistronic) as the CPA gene (mutant or wild type), is shown by stars and was used as an internal control for the efficiency of transfection and expression of each of the expression plasmids.

transfectants were analyzed by SDS-PAGE the wild-type CPA was not processed by any of the mammalian propeptidases, as evident by the absence of the 34-kDa band (Fig. 6A). Interestingly, wild-type pro-CPA was cleaved by the yeast homolog Kex2, as evidenced by the presence of both the 43- and 34-kDa bands. Coexpression of pro-CPA_{95} with PACE, PC7, and Kex2, but not PACE4, also resulted in the appearance of the 34-kDa band representing mature CPA (Fig. 6B). Further, pro-CPA_{95} but not pro-CPA was also processed by PC2 and PC1/3 (data not shown). A spectrophotometric assay for CPA activity using a synthetic substrate, FAPP, demonstrated that in all cases the appearance of mature CPA at 34 kDa was directly correlated with the appearance of CPA enzymatic activity in the conditioned media (data not shown).

To investigate the lack of complete conversion of pro-CPA_{95} to mature CPA in the presence of PACE we tested the hypothesis that PACE expression was limiting. Cotransfections were per-

formed where the ratio of substrate to enzyme was altered by changing the ratio of the expression plasmids for CPA_{95} and PACE. Forty-eight hours posttransfection the cells were labeled with [^{35}S]Met/Cys and the conditioned media were resolved by SDS-PAGE followed by autoradiography (Fig. 7). The level of PACE expression was visualized by Western blot, using an anti-PACE polyclonal antibody (Fig. 7). Pro-CPA_{95} and mature CPA_{95} were detected in the conditioned medium irrespective of the ratio of CPA_{95} to PACE expression plasmids used, except for the leftmost lane in Fig. 7, where CPA_{95} was transfected in the absence of PACE. Quantitation of the percentage conversion of pro- to mature CPA_{95} using phosphoimager analysis (adjusted for the number of Met and Cys residues in pro- and mature CPA_{95}) indicated no significant change in the level of conversion despite increasing levels of PACE. Saturation of wild-type pro-CPA with PACE even to a level of 30:1 still resulted in no conversion of pro-CPA to CPA (data not shown).
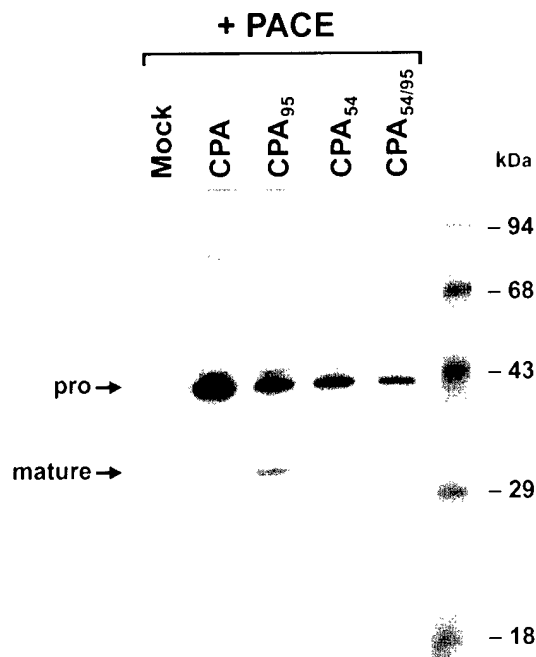
+ PACE

FIG. 5. The mammalian propeptidase PACE can cleave CPA$_{95}$ and CPA$_{54/95}$ but not CPA or CPA$_{54}$. The expression vectors for PACE alone (Mock) or for CPA, CPA$_{95}$, CPA$_{54}$ and CPA$_{54/95}$ were transfected into COS-1 cells and 48 hr post-transfection, the cells were metabolically labeled for 30 min and chased for 4 hr. The conditioned media were then resolved by 12% SDS-PAGE followed by autoradiography. The 43-kDa pro-CPA and the 34-kDa mature CPA bands are indicated.

Purification and enzymatic analysis of CPA$_{95}$

To demonstrate that CPA$_{95}$ was enzymatically active COS-1 cells were transfected with the wild-type or CPA$_{95}$ expres-

sion plasmids alone or in the presence of an expression plasmid for PACE. Serum-free conditioned media were harvested after a 24-hr collection and used for SDS-PAGE and activity assays. Pretreatment of these conditioned media with trypsin prior to electrophoresis resulted in the disappearance of the 43-kDa band and the appearance of a higher mobility band at 34 kDa (Fig. 8A). In addition, the 34-kDa band, present when CPA$_{95}$ was expressed in the presence of PACE, comigrated with trypsin-activated CPA or CPA$_{95}$. When PACE activated CPA$_{95}$, present as both the 43- and the 34-kDa bands, was further treated with trypsin a single polypeptide of 34 kDa was detected, indicating that PACE cleavage was occurring at the authentic site (Fig. 8A). All samples also contained a higher molecular weight band with an apparent mass of 70 kDa that probably results from bovine serum albumin, which is present in the growth medium. Conditioned media from mock-, CPA, and CPA$_{95}$-transfected cells had no detectable carboxypeptidase activity. In contrast, conditioned media from cells cotransfected with CPA$_{95}$ and PACE expression plasmids cleaved MTX-Phe to MTX, as did conditioned media from CPA- or CPA$_{95}$-expressing cells that were trypsinized prior to activity assays (Fig. 8B and C).

To perform more detailed enzyme kinetic analysis we purified trypsin-activated CPA and CPA$_{95}$ as well as PACE-activated CPA$_{95}$. The mature proteins were isolated using a carboxypeptidase potato inhibitor (CPI) affinity column (Ager and Hass, 1977). The recovery of purified mature CPA from the CPI affinity column was ~40% as determined by enzyme assay, and the yield was 1.5–3.0 μg/ml of conditioned medium from transiently transfected of COS-1 cells. SDS-PAGE analysis of pooled fractions from each step of the CPI-based purification protocol is displayed in Fig. 9A. SDS-PAGE analysis also demonstrated that trypsin-activated CPA comigrates with purified CPA$_{95}$ that has either been trypsin or PACE activated (Fig. 9B). Enzymatic analysis of these purified preparations using FAPP (1×10^{-5} to 5×10^{-4} M) demonstrated that trypsin-activated CPA and both trypsin or PACE-activated CPA$_{95}$ have
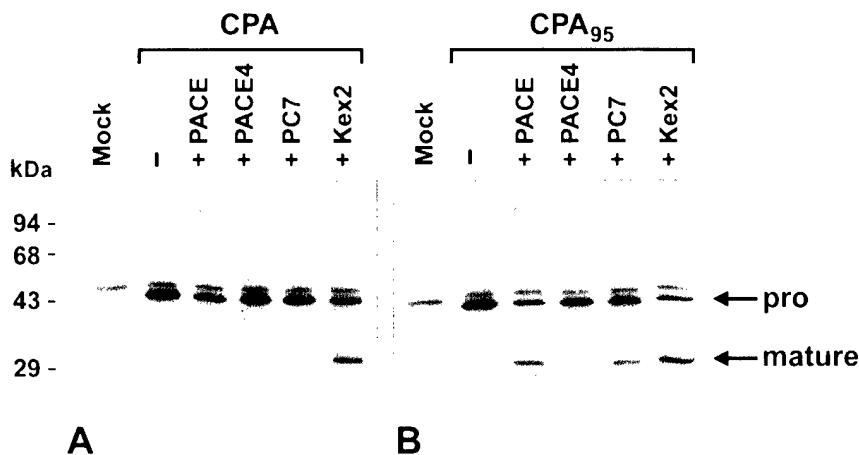

FIG. 6. Processing of CPA and CPA$_{95}$ by other members of the subtilisin-like propeptidase family. COS-1 cells were either mock transfected (Mock) or transfected with the expression vectors for CPA (**A**) or CPA$_{95}$ (**B**) in the absence (−) or the presence (+) of various subtilisin propeptidases. Forty-eight hours after transfection the cells were metabolically labeled and chases for 4 hr, at which time the conditioned media were harvested and analyzed by SDS-PAGE and autoradiography. The 43-kDa pro-form and the 34-kDa mature form of each polypeptide are shown by an arrow.
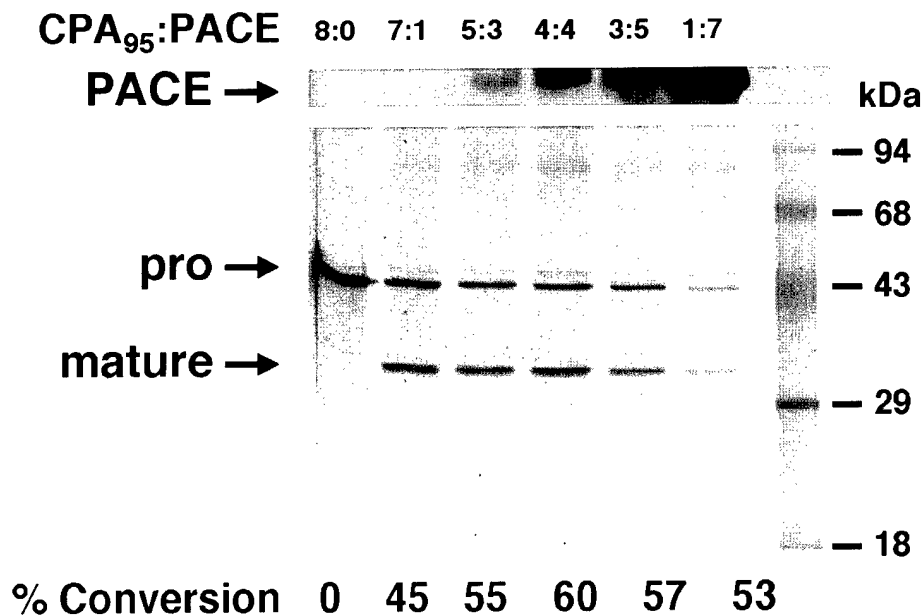
FIG. 7. Processing efficiency of pro-CPA$_{95}$ is similar at various levels of PACE expression. COS-1 cells were transfected with the CPA$_{95}$ expression construct alone (8:0) or in the presence of the PACE expression construct in various proportions (ratio of CPA$_{95}$ to PACE, 7:1, 5:3, 4:4, 3:5, and 1:7). *Top*: A Western blot performed on the cell extracts under each of the preceding conditions, using a PACE-specific polyclonal antibody. *Bottom*: Conditioned media from metabolically labeled cells from the same transfections that were analyed by SDS-PAGE and autoradiography. The 110-kDa PACE-immunoreactive band is labeled, as are the 43-kDa pro- and 34-kDa mature forms of CPA$_{95}$. The amount of radioactivity in the pro- and mature forms of the protein was determined by PhosphoImager analysis and used to calculate the percentage conversion of pro- to mature form under each condition and is shown at the bottom of each lane.

an identical kinetic profile over the range of concentrations studied (Fig. 10). The k_{cat} values determined for FAPP were similar for trypsin-activated CPA or CPA$_{95}$ or PACE-activated CPA$_{95}$ (12,500–13,000 min^{-1}) (Table 1). The K_m values (7.6–8.5 × 10^{-5} M^{-1}) and specific activity (105–120 mU/mg) were also almost identical.

In vitro *cytotoxicity*

Having ascertained that in the absence of trypsinization we could produce mature CPA$_{95}$ that was catalytically indistinguishable from the wild-type enzyme, we set about evaluating the efficacy of the enzyme/prodrug strategy *in vitro*. SCCVII, a mouse squamous cell carcinoma cell line, and UMSCC6, a human squamous cell carcinoma cell line, were transfected with an expression plasmid encoding CPA$_{95}$ alone or were cotransfected with both CPA$_{95}$ and PACE.sol expression plasmids. Stable transfectants were selected on the basis of their resistance to G418, characterized by Western blot, and used for subsequent cytotoxicity assays (Fig. 11). Expression of CPA$_{95}$ without PACE.sol coexpression led to secretion of CPA$_{95}$ predominantly in the inactive form; however, coexpression of both CPA$_{95}$ and PACE.sol led to ~50% conversion of CPA$_{95}$ to mature CPA that was coincident with the appearance of CPA catalytic activity in the conditioned media (data not shown). Parental cells or cells overexpressing CPA$_{95}$ and PACE.sol had similar sensitivities to MTX, with an IC$_{50}$ of 5 nM for SCCVII cells and 25 nM for UMSCC6 cells. MTX-Phe was significantly less toxic than MTX, with an IC$_{50}$ of >10,000 nM for parental

SCCVII cells and 3500 nM in parental UMSCC6 cells. Most importantly, cells expressing both CPA$_{95}$ and PACE.sol were potently sensitized to MTX-Phe, with an IC$_{50}$ of 40 nM for SCCVII cells and 150 nM for UMSCC6 cells. This represents a 25- to 250-fold increase in cytotoxicity, which should allow for a significant therapeutic index *in vivo*.

DISCUSSION

In its normal maturation, pro-CPA requires trypsin-dependent zymogen activation within the lumen of the small intestine. However, in order to use the CPA1 gene in a GDEPT-based application it is necessary to circumvent this trypsin dependence since trypsin is synthesized only by the exocrine pancreas and would not be present within the tumor milieu. Further, owing to its potent and relatively indiscriminant proteolytic activity ectopic experssion of trypsin in a locale other than the small intestine would be expected to be extremely toxic. Previously it had been reported that the propeptide of the pancreatic carboxypeptidases is essential for the proper folding and secretion of the CPAs, and that active CPA cannot be synthesized without its propeptide even if the propeptide is provided in trans (Phillips and Rutter, 1996). We therefore hypothesized that pro-CPA could be altered such that the propeptide would be removed by the proteolytic action of resident subtilisin-like propeptidases that are present within the secretory pathway.

Transfection of COS-1 cells with the expression vectors for wild-type or mutant rat CPA1 constructs resulted in the synthesis of a 43-kDa polypeptide that is consistent with the predicted molecular weight and the reported mass of the protein. The ability of an anti-bovine CPA1 polyclonal antiserum to recognize the recombinant protein further supports the conclusion that the 43-kDa polypeptide encoded by the expression vectors was indeed CPA1. Analysis of conditioned media after metabolic labeling of cells transfected with expression constructs for wild-type CPA and CPA$_{95}$ indicated that the 43-kDa gene product was efficiently secreted into the conditioned media. In contrast, CPA$_{54}$ and CPA$_{54/95}$ were inefficiently secreted while

CPA$_{85}$ and CPA$_{85/95}$ were not detected in the conditioned media. The fact that all of the expression plasmids yielded similar levels of the 43-kDa primary translation product indicates that decreased secretion of CPA$_{54}$, CPA$_{54/95}$, CPA$_{85}$, and CPA$_{85/95}$ was due to intracellular retention. Intracellular retention of proteins is commonly observed when the introduction of mutant residues results in inappropriately folded proteins (Phillips and Rutter, 1996). On the basis of this we predict that introduction of the RARARR (CPA$_{54}$ and CPA$_{54/95}$) sequence in place of the RDAARA sequence, as well as introduction of the RARARR (CPA$_{85}$ and CPA$_{85/95}$) sequence in place of the HGIS sequence, resulted in incorrectly folded proteins, with the
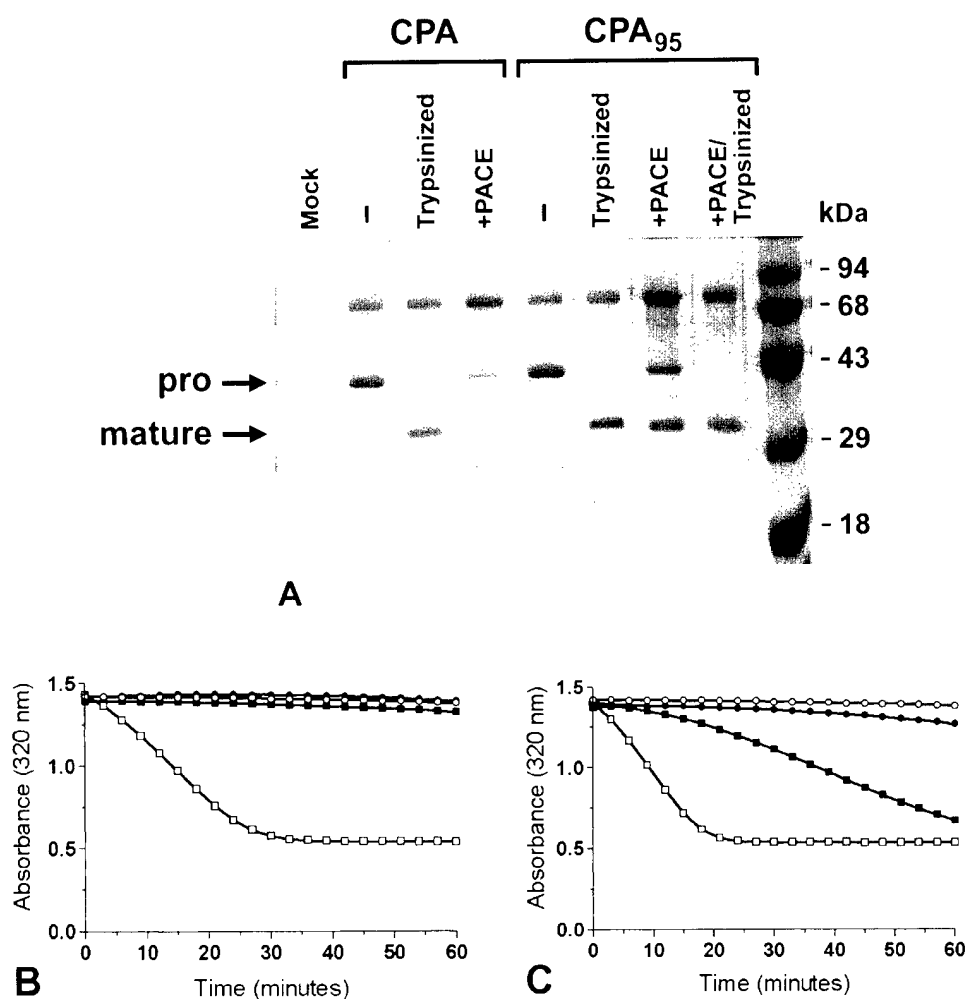

FIG. 8. Processing of CPA$_{95}$ by PACE produces a catalytically active protein. Serum-free conditioned media were harvested from COS-1 cells that were either mock transfected (Mock) or transfected with the CPA or CPA$_{95}$ expression constructs either alone (−) or in the presence of PACE expression plasmid (+PACE). Some samples were trypsin treated prior to analysis (Trypsinized). **(A)** Aliquots of conditioned medium (200 μl) from transfected COS-1 cells were TCA precipitated and analyzed by SDS-PAGE followed by Coomassie blue staining. The 43-kDa pro-form and the 34-kDa mature form of each polypeptide are shown by arrows. Hydrolysis of MTX-Phe by CPA **(B)** and CPA$_{95}$ **(C)** was assayed using conditioned media from COS-1 cells: mock transfected (○), transfected with CPA or CPA$_{95}$ (●), following trypsinization (□), or CPA and CPA$_{95}$ cotransfected with PACE (■). MTX-Phe hydrolysis using 25 μl of the indicated conditioned medium was monitored at 320 nm in assay buffer (pH 7.4, 25 mM Tris-HCl, 25°C, and 0.020 units of carboxypeptidase (G$_2$). Absorbance is plotted as a function of time (min).
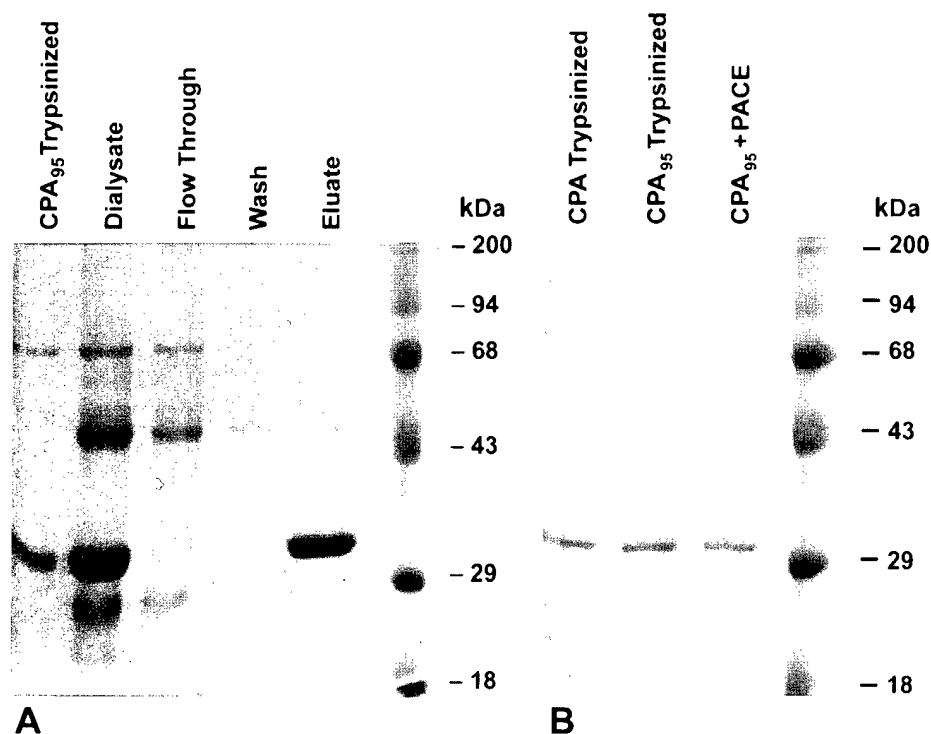
FIG. 9. Purification of mature CPA by affinity chromatography. **(A)** Serum-free conditioned medium was collected from COS-1 cells that were transfected with the expression plasmid for CPA$_{95}$. Prior to purification the conditioned medium was trypsin treated (Trypsinized), dialyzed under vacuum against the binding buffer (Dialysate), and applied to a CPI affinity column, and the flow through collected (Flow Through). The column was washed at neutral pH and low salt (Wash), and finally eluted at alkaline pH and high salt (Eluate). Two hundred microliters from each stage of a representative purification was TCA precipitated and analyzed by SDS-PAGE followed by Coomassie blue staining. **(B)** Five micrograms of each of the purified preparations: trypsin-treated CPA (CPA Trypsinized), trypsin-treated CPA$_{95}$ (CPA$_{95}$ Trypsinized), or PACE-activated CPA$_{95}$ (CPA$_{95}$ + PACE) was analyzed by SDS-PAGE followed by Coomassie blue staining.

latter mutant being more severe since it was not detected in the conditioned media and was rapidly degraded intracellularly (data not shown).

The crystal structure of procine CPA has been determined at 2.0-Å resolution, and from this structure the intramolecular contacts between the propeptide and the catalytic domain have been mapped (Guasch *et al.,* 1992). By extension to rCPA this model does not predict any critical interactions that were altered in the CPA$_{54}$ mutant. However, the adjacent series of amino acids from Asp-47 through Arg-50 (DFWR) in rat CPA are highly conserved among the mammalian pancreatic CPAs, and all four of these residues have been shown to form close interactions between the propeptide and the catalytic domain in porcine CPA. Therefore, it seems that although no coordinating residues were altered in the CPA$_{54}$ mutant the structure of the protein was altered sufficiently to partially inhibit secretion. Similarly, there were no known critical contact residues between the propeptide and the catalytic domain in any of the mutated residues in the CPA$_{85}$ mutant, but the nonconservative nature of the mutations (HGIS) to (**RRRR**) may have disrupted the protein structure enough to cause retention and degradation. In the CPA$_{95}$ mutant only two amino acids were changed (FQAR) to (**RQKR**), and neither of the mutated residues is conserved
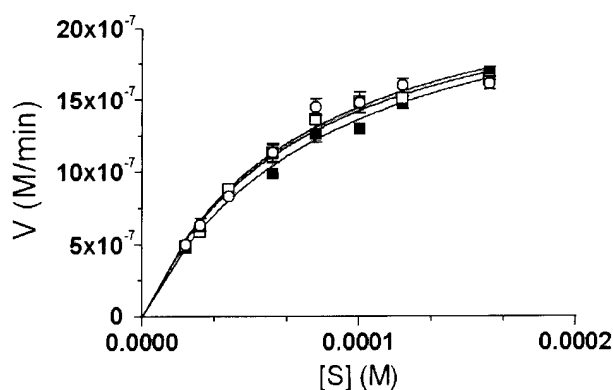

FIG. 10. Hydrolysis of FAPP by CPA. Hydrolysis of FAPP by 2.0×10^{-10} mol of trypsin-activated CPA (○), trypsin-activated CPA$_{95}$ (□), or PACE-activated CPA$_{95}$ (■) was monitored at 330 nm in assay buffer (pH 7.5, 50 mM Tris-HCl, 0.45 M NaCl, 25°C). The means of three separate experiments are shown, where error bars represent the standard error of the mean. Initial velocity V (M/min) was plotted as a function of the substrate concentration [S] (M).

TABLE 1. KINETIC ANALYSIS OF MUTANT AND WILD-TYPE CARBOXYPEPTIDASE A[a]

Enzyme	K_m (1/M)	kcat (1/min)	Specific activity (mU/mg)
CPA trypsinized	$7.8 \times 10^{-5} \pm 9.2 \times 10^{-6}$	$13,000 \pm 725$	105.4 ± 7.4
CPA$_{95}$ trypsinized	$7.6 \times 10^{-5} \pm 7.1 \times 10^{-6}$	$12,500 \pm 550$	115.0 ± 3.4
CPA$_{95}$ 1 PACE	$8.5 \times 10^{-5} \pm 8.4 \times 10^{-6}$	$12,750 \pm 600$	119.9 ± 5.6

[a]Hydrolysis of FAPP by 2.0×10^{-10} mol of trypsin-activated CPA, trypsin-activated CPA$_{95}$, or PACE-activated CPA$_{95}$ was monitored at 330 nm in assay buffer (pH 7.5, 50 mM Tris-HCl, 0.45 M NaCl, 25°C). Kinetic constants were calculated by nonlinear regression and are given as the means of three trials $\pm$ the standard error of the mean. Specific activity was determined at 8.0×10^{-5} M FAPP using bovine CPA as a standard.

across the mammalan pancreatic CPAs; in turn the mutation of these residues had little or no impact on the secretion of CPA$_{95}$.

Expression of CPA$_{95}$ in COS-1 cells did not result in significant levels of activation by endogenous propeptidases. However, we have previously demonstrated that transient expression of natural substrates for the subtilisin-like propeptidases in COS-1 cells does not yield significant processing (Rehemtulla and Kaufman, 1992b); as such COS-1 cells offer a unique environment wherein the expression of the CPA constructs can be monitored in the absence and presence of subtilisin-like propeptidases. However, previous work has also demonstrated that the expression of endogenous substrates in other cell types (e.g., Chinese hamster ovary cells) has led to complete conversion even when no conversion was witnessed in COS-1 cells. Coexpression of CPA$_{95}$ but not CPA$_{54}$ or CPA with PACE resulted in the cleavage of the proprotein (43 kDa) to the mature form (34 kDa). Further confirmation for the appropriate cleavage event was evidenced by the fact that the presence of the 34-kDa band correlated with the appearance of carboxypeptidase activity as determined by MTX-Phe or FAPP cleavage.

The double mutant CPA$_{54/95}$ was processed to a similar extent as CPA$_{95}$, confirming that the PACE cleavage site at 54 was not utilized. The ready cleavage of the arginine residue at amino acid 95 by trypsin and by Kex2 in wild-type CPA, and the cleavage of the PACE recognition site introduced at amino acid 95, suggest that this sequence may be highly accessible and in a conformation that favors cleavage. In contrast, despite the introduction of a PACE cleavage site at residue 54, no cleavage was detected by any of the mammalian propeptidases. This suggests that the CPA$_{54}$ site may not be surface accessible, or it is in a conformation not favorable for cleavage. By constructing the CPA$_{54/95}$ mutant we had predicted that the initial cleavage at Arg-95 would make the arginine at position 54 more accessible, thus resulting in more efficient conversion of pro-CPA to mature CPA. Such sequential cleavage may also be important for trypsin-dependent activation of CPA (Vendrell et al., 1990; Oppezzo et al., 1994). Unfortunately, the double mutant was not processed significantly better than CPA$_{95}$. Given the fact that processing by the subtilisin-like propeptidases occurs later in the secretory pathway, within the trans-Golgi net-
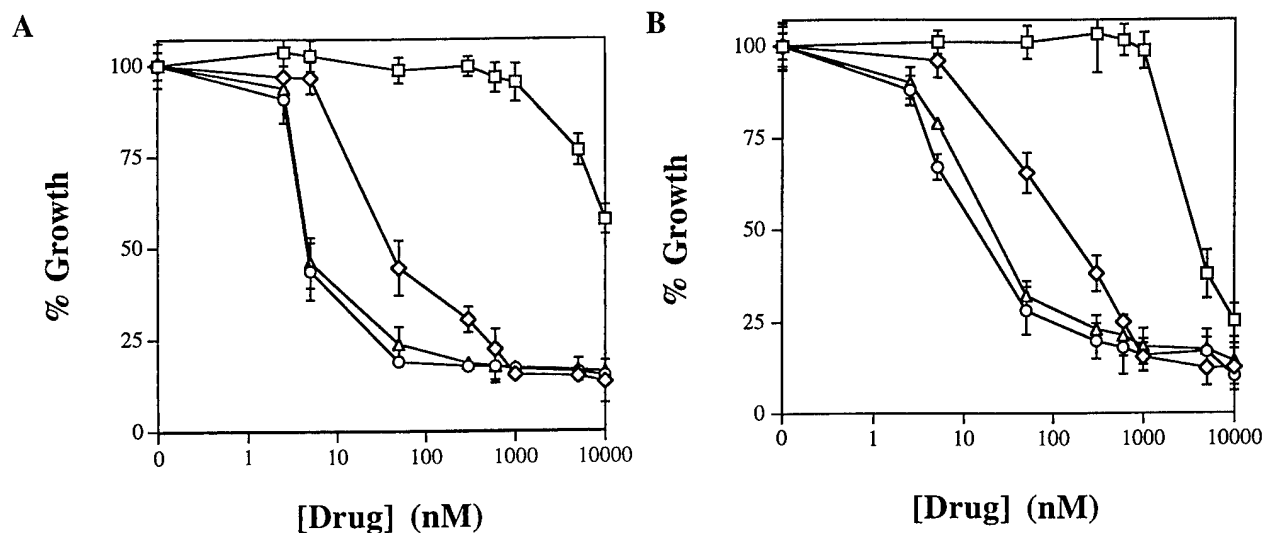

FIG. 11. Cytotoxicity of MTX-Phe to parental cells and cells coexpressing CPA$_{95}$ and PACE. The cytotoxicity of MTX and MTX-Phe was evaluated in two squamous cell carcinoma cell lines using the SRB assay (see Materials and Methods). Parental cells treated with MTX (O), CPA$_{95}$/PACE-coexpressing cells treated with MTX (△), parental cells treated with MTX-Phe (□), and CPA$_{95}$/PACE-coexpressing cells treated with MTX-Phe (◇). (A) SCCVII cells; (B) UMSCC6 cells.

work (Rehemtulla et al., 1992), it is unlikely that the CPA_{85} and $CPA_{85/95}$ mutants, which were retained within the cell (probably in the endoplasmic reticulum), would ever come into contact with the propeptidases. Therefore, it is not surprising that cleavage of the CPA_{85} and $CPA_{85/95}$ mutants was not observed (data not shown).

Coexpression of CPA_{95} with other members of the subtilisin-like propeptidases: PC1/3, PC2, (data not shown) PC7, and Kex2 also resulted in cleavage to the 34-kDa form and the appearance of carboxypeptidase activity. The expression of PC1/3 and PC2 has been detected only in neuroendocrine tissues (Rehemtulla and Kaufman, 1992a); however, both PACE and PC7 have been shown to be ubiquitously expressed (Rehemtulla and Kaufman, 1992a; Seidah et al., 1996). Interestingly, wild-type CPA, as expected, was not cleaved by any of the mammalian propeptidases, but it was cleaved by the yeast enzyme Kex2. Kex2 is known to have a much less stringent recognition site for cleavage than do the mammalian subtilisin-like propeptidases (Rehemtulla and Kaufman, 1992a), which may explain its processing of wild-type rCPA. In addition, the activation of rCPA by Kex2 may also identify Kex2 as the endogenous protease responsible for the partial processing of rCPA when expressed in yeast as previously described (Gardell et al., 1985).

To address the fact that a significant fraction of CPA_{95} remained in the uncleaved proform, we investiagted if the level of PACE was limiting. By titrating the ratio of the CPA_{95} expression vector and the PACE expression vector a spectrum of ratios of the enzyme to substrate was generated. Under all conditions, processing of CPA_{95} by PACE was approximately 50%, indicating that a fraction of the pro-CPA_{95} was refractory to cleavage despite the presence of a PACE cleavage site and excess enzyme. Treatment of this mixture of pro- and PACE-cleaved mature CPA with trypsin resulted in a single polypeptide of 34 kDa with approximately twice the carboxypeptidase activity of the untreated control (data not shown). This demonstrates that the PACE-activated and trypsin-activated proteins are cleaved at the same site (since they comigrate) and that the fraction of CPA_{95} that is refractory to PACE cleavage is still cleavable by trypsin, resulting in a catalytically active protein. Various studies have shown that the cleavage of unprocessed proproteins can occur in the conditioned media after secretion (Rehemtulla and Kaufman, 1992b; Schlokat et al., 1996). To investigate if the lack of complete processing of CPA_{95} from pro- to mature CPA during secretion was due either to compartmentalization or to rapid transit through the trans-Golgi network we coexpressed a soluble, secreted form of PACE (PACE.sol) with CPA_{95}. Under these conditions no improvement in cleavage of CPA_{95} was observed (data not shown), further suggesting that incomplete cleavage was not due to limiting enzyme, limiting time for cleavage, or differential compartmentalization but may be due to two conformational forms of CPA_{95}, one that was accessible to PACE cleavage and one that was refractory.

Owing to the presence of this population of CPA_{95} that was refractory to activation by PACE, we wanted to ascertain whether the fraction of CPA_{95} that was PACE activated had similar kinetic properties when compared with trypsin-activated CPA or CPA_{95}. Mature CPA or CPA_{95} was isolated by CPI affinity chromatography on the basis of the specific binding of CPI to the active site of the enzyme. This binding is blocked by the presence of the propeptide, so pro-CPA_{95} could be separated from mature CPA_{95} for analysis of FAPP cleavage. Kinetic analysis of wild-type CPA and CPA_{95}, whether PACE or trypsin activated, indicated that all three of these forms had indistinguishable catalytic profiles and that the k_{cat} and K_m determined were similar to those obtained for both bovine and porcine CPA (Peterson et al., 1982). Thus, the mutations made in CPA_{95} did not measurably alter the catalytic function of the enzyme.

Having ascertained that CPA_{95} was catalytically active and that PACE-activated CPA_{95} is enzymatically indistinguishable from trypsin-activated CPA, the CPA/MTX-Phe enzyme/prodrug strategy was evaluated in vitro using a cell culture assay. Interestingly, unlike what we had seen with natural endogenous PACE substrates CPA_{95} was not efficiently cleaved when overexpressed in cells in culture (CHO-K1, MCF7, SCCVII, or UM-SCC6; data not shown). However, when cells were selected that expressed both CPA_{95} and PACE.sol, conversion of pro-CPA_{95} to mature CPA was seen in a fashion that was analogous to that witnessed in COS-1 cells. Further, this appearance of mature CPA was coincident with CPA catalytic activity and sensitization to MTX-Phe by a factor of 25- to 250-fold. In this system, sensitization to MTX-Phe occurred only when there was coexpression of both the enzyme (CPA_{95}) and its activator (PACE.sol); however, the use of a bicistronic expression vector should easily allow for this coexpression in an in vivo setting (Davies and Kaufman, 1992).

The CPA-based GDEPT system described here has many potential advantages in the treatment of squamous cell cancer of the head and neck (SCCHN). Currently the treatment of chioce for patients with locally advanced SCCHN is surgery followed by postoperative radiotherapy. For patients with medically inoperable unresectable disease radiotherapy alone is the standard treatment; however, results of such treatment are unsatisfying, with a 5-year survival rate of less than 25%, and most treatment failures occur locally. Since only 10% of patients with SCCHN develop metastatic disease (Merino et al., 1977), it is clear that the development of improved nonsurgical treatments for locally advanced disease would represent a significant advance in the treatment of unresectable/inoperable locally advanced SCCHN. MTX is a first-line chemotherapeutic in the treatment of SCCHN; however, it is limited by systemic toxicities. Therefore, the use of this enzyme/prodrug strategy for cancer therapy of SCCHN has the potential to generate high intratumoral concentrations of MTX without the associated systemic toxicities.

Further, this enzyme/prodrug strategy has many advantages when compared with other strategies that have been proposed. Unlike the HSV TK/ganciclovir (Culver et al., 1992), cytosine deaminase/5-fluorocytosine (CD/5FC; Huber et al., 1994), or carboxypeptidase G_2 (CPG_2)/mustard prodrug strategies (Marais et al., 1996), which all rely on either bacterial or viral proteins, the CPA/MTX-α-peptide strategy utilizes an endogenous protein that is therefore nonimmunogenic. This lack of immunogenicity should allow for long-term expression in tumor cells and for repeated administration when necessary without the associated immunological response. In addition, since gene delivery to tumors in vivo does not lead to transduction of 100% of the cells (Ram et al., 1993; Huber et al., 1994), a vital component of any GDEPT is the presence of a bystander effect,

whereby conversion of prodrug by one transduced cell can kill many other nontransduced neighboring cells (Freeman et al., 1993). The HSV TK/ganciclovir system, with its dependence on gap-junctional intercellular communication (GJIC) for a bystander effect, is limited in this aspect (Fick et al., 1995; Mesnil et al., 1996; Wygoda et al., 1997). Although the CD/5FC strategy exhibits GJIC independence it is limited by the fact that CD is an intracellular enzyme and thus results in the production of high intracellular concentrations of the drug (5FU) resulting in selective killing of transduced cells (Lawrence et al., 1998). CPA1 is an extracellular enzyme and generates MTX from the prodrug within the tumor milieu, thus exposing transduced and bystander cells to an equal concentration of drug in a manner that is not dependent on GJIC. An additional advantage for the use of CPA1 as an enzyme/prodrug strategy is that other antifolates can be used as substrates, such as the α-peptide conjugates of thymidylate synthase inhibitors (Springer et al., 1996). Further studies will focus on the development of a next generation of mutant CPA that is fully activated in the absence of PACE cotransfection and the development of viral vectors that will be used for the delivery of mutant forms of CPA to tumor cells in order to test this enzyme/prodrug system in an animal model.

ACKNOWLEDGMENTS

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Expression of Endogenously Activated Secreted or Cell Surface Carboxypeptidase A Sensitizes Tumor Cells to Methotrexate-α-Peptide Prodrugs[1]

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ABSTRACT

Methotrexate (MTX) is one of the most commonly used agents in the treatment of solid malignancies; however, the toxicities of MTX to bone marrow and gastrointestinal tract complicate this therapy. We, therefore, propose a gene-dependent enzyme prodrug therapy to limit these toxicities by localizing the production of MTX to the site of the tumor. The combination of MTX-α-peptide prodrugs, which cannot be internalized by the cellular reduced folate carrier, with carboxypeptidase A (CPA), which can remove the blocking peptide, has been demonstrated previously *in vitro* using antibody-dependent enzyme prodrug therapy. CPA is normally synthesized as a zymogen that is inactive without proteolytic removal of its propeptide by trypsin. Therefore, to adapt this system to gene-dependent enzyme prodrug therapy, a mutant form of CPA was engineered, CPA_{ST3}, that does not require trypsin-dependent zymogen cleavage but is instead activated by ubiquitously expressed intracellular propeptidases. Purification, peptide sequencing, and kinetic analysis indicated that mature CPA_{ST3} is structurally and functionally similar to the trypsin-activated, wild-type enzyme. In addition, CPA_{ST3}-expressing tumors cells were sensitized to MTX prodrugs in a dose- and time-dependent manner. To limit diffusion of CPA, a cell surface localized form was generated by constructing a fusion protein between CPA_{ST3} and the phosphatidylinositol linkage domain from decay accelerating factor. SDS-PAGE and flow cytometric analysis of infected tumor cells indicated that CPA_{DAF} was cell surface localized. Finally, after retroviral transduction, this enzyme/prodrug strategy exhibited a potent bystander effect, even when <10% of the cells were transduced, because extracellular production of MTX sensitized both transduced and nontransduced cells.

INTRODUCTION

Chemotherapeutics, drugs that are preferentially toxic to tumor cells as compared with host tissues, are a vital part of most current cancer treatments. However, most common chemotherapeutic agents have a small therapeutic index and exhibit profound systemic toxicities, particularly to rapidly dividing tissues such as bone marrow and gastrointestinal tract (1). These toxicities present a significant morbidity and mortality; in addition, they limit the dose of chemotherapeutic used and thus may also decrease the clinical response. One method proposed to circumvent these toxicities and to increase the therapeutic index of chemotherapy is the development of GDEPT[3] (reviewed in Ref. 2): (*a*) tumors are transduced with the gene for an enzyme whose activity is not normally present in the host; and (*b*) a prodrug is administered systemically, which is nontoxic except when metabolically converted to a toxic form by the enzyme transduced in the first step. The goal of these strategies is to simultaneously increase the local concentration of the toxic agent while also decreasing the associated systemic toxicities.

MTX, a folate analogue antimetabolite, is one of the most commonly used chemotherapeutics for the treatment of solid malignancies (3, 4). Prodrugs of MTX have been described where a blocking amino acid is conjugated to the glutamic acid residue in MTX; these prodrugs are unable to be internalized by the cellular reduced folate carrier (5). CPA, a zinc-metalloprotease, is normally synthesized in the pancreas and released into the lumen of the small intestine, where trypsin-dependent zymogen activation is necessary to remove the inhibitory propeptide and activate the enzyme (6). CPA has been described previously for use in ADEPT protocols in conjunction with MTX-α-peptide prodrugs (5, 7, 8). All of these ADEPT strategies relied upon purified CPA that was activated by trypsin *in vitro* to remove the propeptide. However, ADEPT systems are plagued by a number of problems, including cost and difficulties with development and purification of antibodies, immunogenicity of antibodies, accessibility of tumor to the enzyme/antibody conjugate, stability of the enzyme/antibody conjugate, and background conversion of prodrugs because of localization of antibody conjugates to inappropriate tissues (2). To adapt this CPA/MTX-α-peptide-based strategy from an antibody-based therapy to a GDEPT, we endeavored to generate mutant forms of CPA that would be activated in a trypsin-independent manner by endogenous cellular proteases.

PACE/furin is the prototypical member of a family of PCs that include at least seven members (9). These serine proteases are involved in the maturation of secretory proteins by cleavage after clusters of basic amino acids in proteins such as: growth factors, growth factor receptors, prohormones, bacterial toxins, and viral coat proteins. Some of the PCs exhibit restrictive expression in neuroendocrine tissues; however, at least three members of the family, PACE, PACE4, and PC7, appear to be ubiquitously expressed (9). Therefore, we felt that a mutant form of CPA, which was engineered to be activated by one or more of these PCs, could prove to be an effective part of a GDEPT strategy. Previously, we have reported just such a mutant, CPA_{95}, where a simple tetra-basic PACE cleavage site (-RQKR-) was introduced into CPA between the propeptide and the mature enzyme (10). CPA_{95} was expressed as an active enzyme independent of trypsin treatment and could sensitize cells to MTX-Phe. This activation, however, was dependent upon overexpression of PACE with little or no activation detected by endogenous PCs in absence of PACE cotransfection. To overcome the need for exogenous PACE expression, we report here a mutant form of CPA (CPA_{ST3}) that is fully activated in a trypsin-independent manner by endogenous propeptidases. The rationale for the construction of CPA_{ST3} was that the 10 amino acid sequence (-GLSARNRQKR-) within ST3 sensitizes it to activation by PACE (11). Because this sequence encompasses the key features of a PACE cleavage site (*i.e.*, basic residues at -1, -2, -4, and -6 relative to cleavage; Ref. 12), we
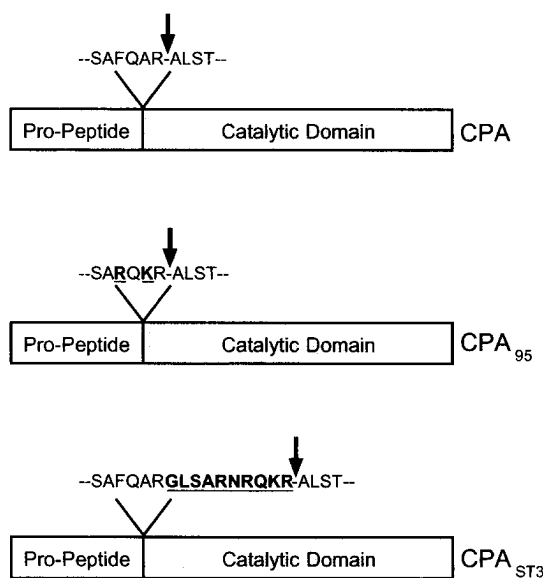
Fig. 1. Mutations introduced into the CPA protein to facilitate subtilisin-like propeptidase cleavage. Wild-type CPA, CPA_{95}, and CPA_{ST3} are depicted in diagrammatic form, mutated residues are *underlined*, and the cleavage sites are indicated with an *arrow*.

hypothesized that the insertion of this decapeptide would also sensitize CPA_{ST3} to PC-based activation.

The secretion of active CPA into the extracellular space should allow for a potent bystander effect where a small population of CPA expressing cells could generate sufficient MTX within the tumor milieu to sensitize adjacent, nontransduced cells. This is particularly true for this enzyme/prodrug system because the MTX is generated outside the transduced cell, so both CPA-expressing and bystander cells should be equally sensitized. Unfortunately, if active secreted CPA is able to diffuse out of the tumor, it might result in both a decreased local production and in increased systemic generation of MTX. Therefore, to restrict CPA to the site of transduction, we constructed a cell surface tethered form of the enzyme by fusing CPA_{ST3} to the glycophospholipid membrane linkage domain of DAF (13). This fusion protein is anchored to the surface of the cell by a lipid linkage and thus may afford local production of MTX without systemic release of the protein. In this report, the generation of both the endogenously active soluble and cell surface forms of CPA are described, as is their ability to use MTX-α-peptide prodrugs and sensitize cells *in vitro*.

MATERIALS AND METHODS

Expression Plasmids. All common molecular biological techniques were performed according to Sambrook *et al.* (14). The expression plasmids for wild-type rat CPA, the single PACE cleavage mutant of CPA at amino acid 95 (CPA_{95}), PACE, or the dominant-negative form of PACE (PACE.SA) have all been described previously (10, 12). The endogenously activated mutant of CPA (CPA_{ST3}) and the cell surface-localized form of this mutant (CPA_{DAF}) were constructed by overlap PCR (15) using wild-type CPA and CPA_{ST3} as template, respectively, and subcloned into the mammalian expression vector pZ (kindly provided by The Genetics Institute, Cambridge, MA). CPA_{ST3} introduced the decapeptide sequence (-GLSARNRQKR-; Ref. 11) between the prodomain and mature domain of rat CPA (Fig. 1), and CPA_{DAF} fused the 37 amino acid region (-PNKGSGTTSGTTRLLSGHTCFTLTGLLGTLVTM-GLLT-) from DAF to the COOH terminus of CPA_{ST3} (Ref. 13; see Fig. 6a). All plasmids were confirmed by sequencing at the University of Michigan DNA Sequencing Core.

Cell Culture and Transfections. All cells were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, penicillin,

streptomycin, and L-glutamine, except for MCF7 cells, which were cultured in RPMI medium with the same supplements. Expression plasmids were transiently transfected into SV40 large T-antigen expressing human embryonic kidney cells (293T) by calcium phosphate precipitation using equal amounts of plasmid DNA for each transfection (10 μg/ml transfection mixture). To generate CPA for purification, enzymatic assays, or Western blotting, 48 h after transfection plates were washed three times with PBS and then incubated in serum-free medium (Optimem; Life Technologies, Inc., Gaithersburg, MD) for an additional 24 h; at which time the supernatants were harvested, nonadherent cells spun down by centrifugation for 15 min at $1000 \times g$, and the conditioned medium frozen at −70°C for subsequent analysis. Stable cell lines expressing CPA_{ST3} were generated by transfecting the CPA_{ST3} expression plasmid or a control plasmid into SCCVII cells using Lipofectamine-PLUS (Life Technologies). Pooled polyclonal transfected cells were then selected for G418 (Life Technologies) resistance for three passages prior to cytotoxicity experiments.

Protein Analysis. For experiments requiring metabolic labeling of proteins, [^{35}S]methionine/cysteine (Pro-mix; Amersham, Arlington Heights, IL) was used according to the protocols described previously (12). Western blotting was performed as described previously (10) using a rabbit polyclonal anti-bovine CPA antisera (Cemicon, Temecula,-CA), followed by enhanced chemiluminescence (Pierce, Rockford, IL).

CPA Purification and Enzymatic Assays. CPA was purified from conditioned medium using CPA potato inhibitor affinity chromatography (10) or an α-CPA immunoaffinity column. The α-CPA affinity column was made according to the manufacturer's instructions (Affi-Gel Hz; Bio-Rad, Hercules, CA) using a rabbit α-bovine CPA antibody. Conditioned media were diluted 1:1 with PBS, loaded onto the column, washed (500 mM NaCl in PBS), and eluted (500 mM NaCl, 20 mM Glycine HCl, pH 2.0). Purified CPA was then dialyzed against 500 mM NaCl, 50 mM Tris-HCl (pH 8.0) and stored at 4°C. CPA activity was measured using a spectrophotometric assay for cleavage of a synthetic substrate, N-(3-[2-furyl]acryoyl)-Phe-Phe (Sigma Chemical Co., St. Louis, MO) as described previously (10). Data were plotted, and kinetic constants were calculated by nonlinear regression using GraphPad Prism (GraphPad Software, San Jose, CA).

Retroviral Production and Infection. The cDNAs for CPA_{ST3} or CPA_{DAF} were subcloned into Lzrs.pBMN (kindly provided by Gary Nolan, Stanford, CA), yielding Lz.CPA_{ST3} and Lz.CPA_{DAF}. To generate retroviruses coding for both CPA_{ST3} or CPA_{DAF} and the neomycin resistance gene (neo^R) from one bicistronic retrovirus using an IRES, the entire CPA_{ST3}/IRES/neo^R or CPA_{DAF}/IRES/neo^R expression cassettes were amplified by PCR and subcloned into Lzrs.pBMN, yielding Lz.Neo.CPA_{ST3} and Lz.Neo.CPA_{DAF}. Retroviruses were produced by transfecting the ΦnX-ampho packaging cell line (kindly provided by Gary Nolan, Stanford, CA) using calcium phosphate precipitation, and 48 h after transfection, the producer cells were selected in 0.5 μg/ml puromycin (Sigma). Retroviral supernatants were generated by plating puromycin-selected producer cells at a density of 40,000 cells/cm^2 in 100-mm plates and culturing at 32°C for 4 days, with daily harvests. At this time, the supernatants were pooled, filtered through a 0.4 μm filter, aliquoted, and frozen at −70°C. Cells were infected using retroviral supernatants in the presence of Polybrene (16 μg/ml; Sigma). The titer of each reach retroviral batch was determined using SCCVII cells and G418 selection for CPA constructs and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining for β-galactosidase constructs. The titers achieved were $2.4 \times 10^6 \pm 0.7 \times 10^6$, $2.5 \times 10^6 \pm 0.6 \times 10^6$, and $1.1 \times 10^6 \pm 0.3 \times 10^6$ colony-forming units/ml for LacZ, CPA_{ST3}, and CPA_{DAF} respectively.

HPLC Analysis. Tissue culture media were acidified with 1/10th volume 1 M HCl and extracted with −20°C CH_3CN (5:2 CH_3CN:media). Extracts were then analyzed on a C_{18} reverse phase column (Waters, Milford, MA) by HPLC on a gradient from 10:90:0.1 to 50:90:0.1 CH_3CN:H_2O:trifluoroacetic acid at a flow rate of 1 ml/min over 15 min. HPLC was performed on a Waters gradient system composed of two model 501 pumps, a U6K injector module, and a model 996 photodiode array detector; the system was controlled by Millennium 2010 software. Absorbance was monitored at 315 nm, and under these conditions, MTX had a retention time of 6.4−6.6 min, and MTX-Phe had a retention time of 8.4−8.6 min.

Flow Cytometry. To evaluate cell surface expression of CPA, SCCVII cells were infected with LacZ, CPA_{ST3}, or CPA_{DAF} retrovirus; 48 h later, they were detached from dishes using trypsin, and the trypsin was inactivated by the addition of serum. Cells were then incubated on ice for 30 min in medium

supplemented with a 1:200 dilution of α-CPA antibody. Subsequently, they were centrifuged through a 1/2 ml of FBS to isolate them from unbound antibody and then resuspended in medium supplemented with a 1:200 dilution of R-phycoerythrin conjugated goat α-rabbit IgG secondary antibody (Fischer, Pittsburgh, PA). After 30 min, the unbound antibody was again removed, and the cells were resuspended in PBS for analysis at the University of Michigan Flow Cytometry Core.

Cytotoxicity Assays. For some experiments, growth inhibition was assayed using the sulforhodamine B assay (Sigma; Ref. 16). Cells were plated at a density of 3000 cells/cm^2 in a 96-well plate; 12–18 h after plating, cells were infected with retroviral supernatants. Twenty-four h after infection, the medium was changed to that supplemented with vehicle (PBS), MTX, or MTX-α-peptides (17). The cells were left cultured with the drug for 72 h, at which point they were fixed and stained according to Skehan *et al.* (16). Data represent the mean ± SE of at least eight replicate wells. For other experiments, cells were assayed using a CFA (18). Cells were plated in 60-mm dishes at a density of 2000 cells/cm^2; 18–24 h later, they were infected with retroviral supernatants for 24 h, at which time the medium was changed to that supplemented with vehicle (PBS), MTX, or MTX-Phe. Cells were cultured with the drug for the indicated time and then plated for colony formation; and after 7–10 days, the dishes were fixed and stained with crystal violet before counting. Data plotted represent the mean and SE of at least three experiments.

CPA Diffusional Assay. To measure the ability of secreted, soluble CPA to sensitize nontransduced cells, a two-chamber tissue culture plate was used. A 50/50 mixture of CPA$_{ST3}$- or CPA$_{DAF}$-expressing SCCVII cells and parental SCCVII cells (4000 total) were plated into the top wells of a six-well transwell plate (Costar Transwell-clear; Fischer, Pittsburgh, PA), and at the same time, an equal number (4000 cells) of parental SCCVII cells were plated into the bottom chambers. The top and bottom chambers were separated by a permeable membrane with 0.4 μM pores such that CPA or small molecules like MTX or MTX-Phe could freely move between the chambers, but whole cells were prohibited from crossing the barrier. The cells were left seeded in the chamber for 48 h, at which time MTX-Phe was added directly to the top and bottom chambers at a uniform concentration of 1 μM. Both the top and bottom chambers from parallel wells were subsequently trypsinized and plated for CFA at 12-h intervals.

RESULTS

CPA$_{ST3}$ Is Activated by Endogenous Prohormone Convertases. To use CPA in a GDEPT strategy for cancer therapy, we constructed a mutant that is expressed as an active enzyme in the absence of trypsin-dependent propeptide cleavage. Previously, we reported that CPA$_{95}$, a mutant into which a PC cleavage site was introduced by two amino acid substitutions (FQAR → RQKR), is activated but only in the presence of PACE overexpression (Fig. 1; Ref. 10). We, therefore, constructed CPA$_{ST3}$, which includes a 10-amino acid linker region (-GLSARNRQKR-) between the propeptide and mature domain of CPA (Fig. 1), where the underlined amino acids represent a prototypical PACE/furin cleavage site. This linker region is derived from the matrix-metalloprotease ST3, where it has been demonstrated to sensitize ST3 to PC-dependent activation (11).

To examine the expression and processing of CPA$_{ST3}$, 293T cells were transiently transfected with the expression plasmids for wild-type CPA, CPA$_{95}$, or CPA$_{ST3}$ in the absence or presence of a PACE expression plasmid. Conditioned media were collected from transfected cells and analyzed by SDS-PAGE and Western blot. Expression of wild-type CPA alone as well as with cotransfected PACE resulted in a M_r 43,000 protein, which is characteristic of pro-CPA (Fig. 2, *Lanes 2* and *3*). When CPA$_{95}$ was expressed in 293T cells, there was a small amount of mature CPA generated, as evidenced by the band at M_r 34,000, yet the majority of the protein was still in the pro- form (Fig. 2, *Lane 4*). However, in the presence of PACE cotransfection, >50% of CPA$_{95}$ was processed to the mature form, while the rest remained in the larger pro- form (Fig. 2, *Lane 5*). These

results are consistent with previous observations for CPA$_{95}$ when expressed in COS-1 cells and in squamous cell carcinoma lines (10).

In contrast, when CPA$_{ST3}$ was expressed in 293T cells, it was completely processed to the mature form, even in the absence of cotransfected PACE (Fig. 2, *Lane 6*), and cotransfection of PACE had no impact upon this activation and secretion (Fig. 2, *Lane 7*). To verify the specificity of this activation, PACE.SA, a dominant-negative mutant of PACE where the active-site serine was mutated to alanine (12), was cotransfected along with CPA$_{ST3}$. Cotransfection of PACE.SA and CPA$_{ST3}$ inhibited the conversion of CPA$_{ST3}$ from the pro- to mature form (Fig. 2, *Lane 8*), a further indication that activation of CPA$_{ST3}$ was achieved through the action of endogenous PCs. The observed molecular weights of pro- and mature CPA seen here are consistent with those reported previously for trypsin-activated wild-type CPA (10).

Endogenously Activated CPA$_{ST3}$ Is Indistinguishable from Trypsin-activated Wild-Type CPA. 293T cells were transiently transfected with the CPA$_{ST3}$ expression plasmid, and the protein was purified from the conditioned medium using an anti-CPA immunoaffinity column. Enzymatic analysis using a synthetic substrate, N-(3-[2-furyl]acryoyl)-Phe-Phe (1 × 10^{-5} M to 5 × 10^{-4} M), demonstrated that endogenously activated CPA$_{ST3}$ had a similar kinetic profile to trypsin-activated wild-type CPA over the range of substrate concentrations studied with K_m and $kcat$ values, which were virtually identical (Table 1). In addition, conditioned medium from 293T cells transiently transfected with CPA$_{ST3}$ was submitted to the University of Michigan Protein Structure Core for electrophoresis and NH$_2$-terminal sequencing. Ten consecutive amino acids were identified (-ALSTDSFNYA-), which correspond to the first 10 amino acids of mature rat CPA that were immediately COOH-terminal of the PC cleavage site introduced via the ST3 linker region (see Fig. 1; Ref. 19).

Expression of CPA$_{ST3}$ in Squamous Cell Carcinoma Cells Leads to Conversion of MTX-Phe to MTX and Cytotoxicity in a Time-dependent Manner. SCCVII murine squamous cell carcinoma cells were transfected with the pZ.CPA$_{ST3}$ expression plasmid, and a pooled polyclonal CPA$_{ST3}$-expressing population was selected with G418. The conditioned medium from cells expressing CPA$_{ST3}$ contained CPA, which was predominantly in the mature form, as detected by Western blot and activity assay (data not shown and see Fig. 4a). CPA$_{ST3}$- or LacZ-expressing cells were exposed to MTX or MTX-Phe for 0–72 h and then plated to determine the SF (Fig. 3a). In addition, at the time of plating conditioned media were collected for analysis by HPLC (Fig. 3b). When either LacZ- or CPA$_{ST3}$-expressing cells were exposed to 1 μM MTX, there was significant cytotoxicity observed starting 12 h after exposure, which peaked at a SF of <0.001 after 36–48 h (Fig. 3a). However, there was no apparent cytotoxicity to LacZ-expressing cells exposed to 1 μM MTX-Phe for 72 h with a SF > 0.9 (Fig. 3a) and no detectable conversion of MTX-Phe to MTX (data not shown). In contrast, CPA$_{ST3}$-expressing cells were potently sensitized to MTX-Phe; this cytotoxicity approached that of MTX, reaching a SF of slightly >0.001 after 72 h of exposure to 1 μM MTX-Phe (Fig. 3a).

The time course of cytotoxicity for MTX-Phe in CPA-expressing cells was somewhat delayed when compared with MTX toxicity; however, because the CPA in the culture medium was removed when the medium was replaced with fresh medium containing MTX-Phe, this delay in cytotoxicity was probably attributable to the time needed for the cells to synthesize fresh CPA. Indeed, the delayed cytotoxicity directly correlated with the production of MTX in the tissue culture medium, as determined by HPLC (Fig. 3b). Furthermore, the addition of MTX-Phe directly to the culture without changing the medium
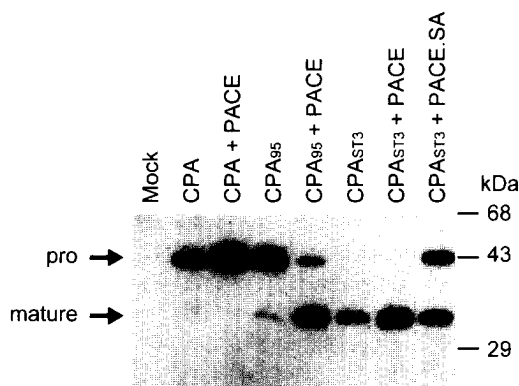
Fig. 2. Expression of CPA$_{ST3}$ results in mature CPA in the absence of PACE coexpression. Expression plasmids for wild-type, CPA$_{95}$, and CPA$_{ST3}$ constructs were transfected into 293T cells either alone or in cotransfection with PACE or dominant-negative PACE (*PACE. SA*). A total of 5 μg of the different CPA expression plasmids were used in each condition supplement with 5 μg of the PACE constructs or empty vector. Forty-eight h after transfection, conditioned media were harvested and analyzed by SDS-PAGE and Western blot analysis using a CPA-specific polyclonal antibody. *Arrows*, pro and mature forms of CPA. As a control, 293T cells were transfected in the absence of DNA and analyzed as above (*Mock*).

Table 1 *Kinetic analysis of wild-type CPA and CPA$_{ST3}$*

Hydrolysis of *N*-(3-[2-furyl]acroyl)-Phe-Phe by 2.0 × 10^{-10} mol trypsin-activated CPA or endogenously activated CPA$_{ST3}$ was monitored at 330 nm in assay buffer (pH 7.5; 50 mM Tris-HCl, 0.45 M NaCl; 25°C). Kinetic constants were calculated by nonlinear regression and are given as the mean of three trials ± SE.

Enzyme	K_m (mM)	kcat (1/min)
CPA	$7.8 \times 10^{-2} \pm 9.2 \times 10^{-3}$	$13{,}000 \pm 725$
CPA$_{ST3}$	$8.2 \times 10^{-2} \pm 9.6 \times 10^{-3}$	$12{,}250 \pm 1{,}135$

shifted the time course of sensitization such that it more closely paralleled that of MTX (data not shown).

Retroviral Transduction of Tumor Cell Lines Leads to Production of Mature CPA and Sensitivity to MTX-Phe. Because the use of stable cell lines may not accurately represent a gene therapy strategy, a CPA$_{ST3}$ retrovirus was produced by subcloning the CPA$_{ST3}$ cDNA into the Laz.pBMN expression plasmid and transfecting the ΦNxa retroviral producer line. A Western blot of the conditioned medium from this retroviral producer line indicated that these cells secrete mature CPA$_{ST3}$ (Fig. 4a). Three tumor cell lines, SCCVII murine squamous cell carcinoma, UMSCC6 human squamous cell carcinoma, and MCF7 human breast carcinoma, were infected with either CPA$_{ST3}$- or LacZ-expressing retrovirus. LacZ infection, followed by β-galactosidase staining, revealed that ~50% of SCCVII cells were infected, whereas for the other two cell lines, the infection rate was ~25–30% (data not shown). Forty-eight h after infection, the cells were labeled with [^{35}S]methionine/cysteine for 30 min, followed by a 4-h chase, and the conditioned media were immunoprecipitated using an α-CPA antibody and then analyzed by SDS-PAGE and autoradiography. Cells infected with LacZ virus did not produce any detectable CPA; however, cells infected with the CPA$_{ST3}$ retrovirus produced CPA that was predominantly in the mature form, as evidenced by the band at M_r 34,000 (Fig. 4a). In addition, in a parallel series of plates, infected cells were exposed to 1 μM MTX-Phe for 72 h before plating to analyze their SF. For all three lines tested, LacZ-infected cells were resistant to MTX-Phe (SF >0.75), whereas CPA$_{ST3}$-infected cells were potently sensitized to the prodrug (SF of 0.01 to 0.00 liter).

CPA$_{ST3}$-expressing Cells Are Sensitized to MTX-Phe in a Dose-dependent Manner. More detailed studies on prodrug activation by CPA$_{ST3}$ were performed using SCCVII cells at a range of MTX and

MTX-Phe concentrations from 1 to 1000 nM. Both LacZ- and CPA$_{ST3}$-expressing cells were sensitive to MTX exhibiting a SF of 0.001 at 1000 nM MTX and IC$_{50}$ and IC$_{95}$ values of 1 and 25 nM, respectively (Fig. 5 and Table 2). There was no toxicity to LacZ-infected cultures, even when exposed to 1000 nM MTX-Phe (Fig. 5 and Table 2). However, infection of SCCVII cells with CPA$_{ST3}$ retrovirus, followed by exposure to MTX-Phe, resulted in cytotoxicity in a dose-dependent manner that paralleled that of MTX exhibiting a SF of about 0.001 at 1000 nM MTX-Phe and IC$_{50}$ and IC$_{95}$ values of 2.5 and 35 nM, respectively (Fig. 5 and Table 2).

Construction and Characterization of a Cell Surface Form of CPA. The GDEPT strategy for cancer therapy described here relies upon the extracellular secretion of soluble active CPA, which can then cleave the prodrug MTX-Phe, yielding MTX. However, release of a secreted and diffusible form of CPA into the extracellular space in an *in vivo* model has the potential to result in both decreased tumoral cytotoxicity and also systemic toxicity. To alleviate these limitations, we constructed a modified form of CPA$_{ST3}$ wherein the COOH terminus of DAF was fused to CPA$_{ST3}$, and the molecule thus was linked to the cell surface by a glycophospholipid linkage (Fig. 6a).

Both the soluble (CPA$_{ST3}$) and the cell-surface tethered form (CPA$_{DAF}$) of CPA were transiently expressed in 293T cells; 48 h after transfection, the cells were labeled with [^{35}S]methionine/cysteine for 10 min, and cell extracts were collected. In parallel plates, labeled cells were chased in serum-free medium for 5 h prior to the collection of both cell extracts and conditioned medium. Cell lysates from both the early and late time points were then lysed in a Dounce homogenizer and submitted to a 100,000 × g spin to precipitate cellular
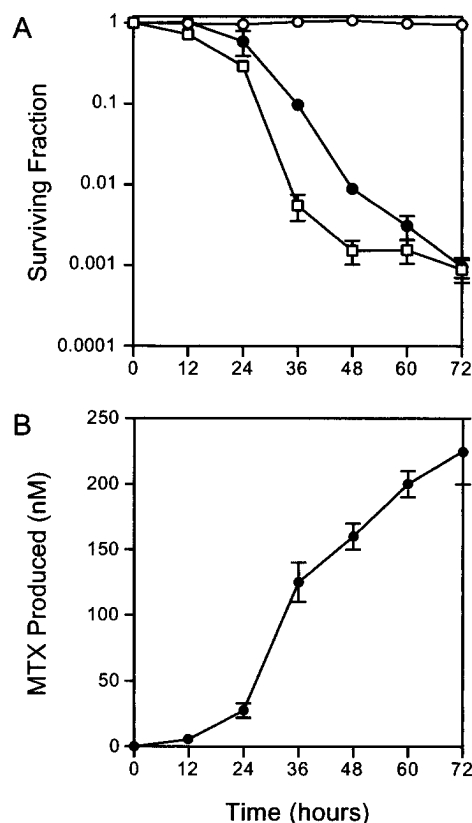

Time (hours)

Fig. 3. CPA$_{ST3}$-expressing cells convert MTX-Phe to MTX and are sensitized to the prodrug in a time-dependent manner. SCCVII cells expressing LacZ or CPA$_{ST3}$ were exposed to MTX or MTX-Phe for 0–72 h and then evaluated by CFA (*A*), and HPLC analysis was performed on the condition media at the time of plating (*B*). LacZ-expressing cells treated with MTX (□) or MTX-Phe (○) and CPA $_{ST3}$-expressing cells exposed to MTX-Phe (●) are shown.
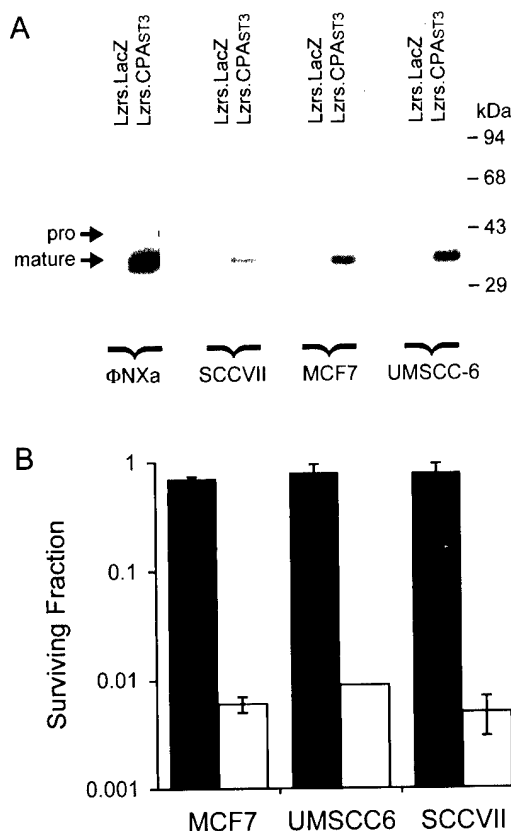
Fig. 4. Retroviral infection with CPA$_{ST3}$ results in secretion of mature CPA and sensitization to MTX-Phe. The supernatants from Lzrs.LacZ or Lzrs.CPA$_{ST3}$ retroviral producer cells were analyzed by SDS-PAGE and Western blotting for CPA expression (A, leftmost panel). Three tumor cell lines (SCCVII, MCF7, and UMSCC6) were infected with LacZ or CPA$_{ST3}$ retrovirus, metabolically labeled with [^{35}S]methionine/cysteine, and conditioned media were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography (A, right three panels). Arrows, pro and mature forms of CPA. In parallel cultures, LacZ or CPA$_{ST3}$-infected cells were treated with MTX-Phe for 48 h and plated for surviving fraction (B). Bars, SE.

infected with CPA$_{DAF}$ had a >100-fold increase in staining using an anti-CPA antibody when compared with LacZ- or CPA$_{ST3}$-infected cells, thus demonstrating that not only is CPA$_{DAF}$ cell associated, but it is also cell-surface exposed (Fig. 6c).

To evaluate whether the CPA$_{DAF}$ molecule retained a substrate specificity similar to the native enzyme, we performed sulforhodamine B growth inhibition assays using five different MTX-α-peptides (17) to compare the substrate specificity of CPA$_{ST3}$ and CPA$_{DAF}$, as measured by their ability to sensitize cells to these prodrugs using the sulforhodamine B growth inhibition assay (Table 3). Both CPA$_{ST3}$ and CPA$_{DAF}$ showed a preference for the large aromatic side chain prodrugs, with MTX-Phe being the best used substrate, followed by MTX-Tyr. Both forms of CPA had slight activity against MTX-Met with little or no activity versus MTX-Gln and MTX-Trp. Although the trend of substrate specificity was consistent between CPA$_{ST3}$ and CPA$_{DAF}$, the absolute level of activity varied with CPA$_{ST3}$ consistently having greater activity than CPA$_{DAF}$; however, this difference may not reflect actual differences in activity and instead most likely is an indication of different titers of retroviruses (see below).

CPA$_{ST3}$ and CPA$_{DAF}$ Both Exhibit a Potent Bystander Effect. In any cancer gene therapy strategy, only a small portion of the tumor can normally be transduced, typically <10% of the total tumor mass. Therefore, the ability of transduced cells to kill both transduced and nontransduced cells is an important aspect of a GDEPT strategy. To evaluate the potential bystander effect of the system described herein, retroviral constructs were generated where either CPA$_{ST3}$ or CPA$_{DAF}$ are expressed from the same bicistronic mRNA as the neomycin resistance gene (neo^R), thus enabling one to select and quantify infected cells based upon resistance to G418. SCCVII cells were infected with titers of Lz.Neo.CPA$_{ST3}$ or Lz.Neo.CPA$_{DAF}$ retrovirus ranging from 1.25e^4 to 1.0e^6 colony-forming units/ml in a series of parallel dishes. In one pair of dishes, cells infected at increasing titers of virus were plated at varying dilutions with and without G418
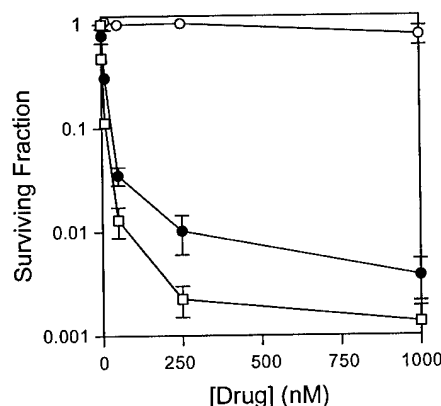
membranes (S100). The S100 fractions as well as the conditioned medium were analyzed by immunoprecipitation, followed by SDS-PAGE and autoradiography. CPA$_{ST3}$ was initially synthesized as a M_r 43,000 pro-form and subsequently converted to the M_r 34,000 mature form and secreted from the cell such that after a 5-h chase, it accumulated in the conditioned medium and was no longer detectable in the cell extract (Fig. 6b). In contrast, CPA$_{DAF}$ was initially synthesized as a M_r 48,000 form and then converted to a M_r 38,000 form, which remained cell associated and was undetectable in the conditioned medium (Fig. 6b), thus indicating that CPA$_{DAF}$ is cell associated whereas CPA$_{ST3}$ is secreted.

In a separate experiment, 293T cells were mock-transfected or transfected with either CPA$_{ST3}$ or CPA$_{DAF}$. Conditioned medium and S100 fractions were collected from the transfected plates and assayed for CPA activity. The S100 cell pellet from mock or CPA$_{ST3}$-transfected cells had little or no catalytic activity, whereas the S100 fraction from CPA$_{DAF}$ transfected cells rapidly cleaved the synthetic substrate (data not shown). Finally, unlike CPA$_{ST3}$ which contained significant catalytic activity in the conditioned medium, such activity was undetectable in the conditioned medium derived from mock or CPA$_{DAF}$-transfected cells (data not shown).

To further verify that CPA$_{DAF}$ was expressed on the cell surface, a CPA$_{DAF}$ retrovirus was generated by subcloning the cDNA for CPA$_{DAF}$ into Laz.pBMN. SCCVII cells were infected with LacZ, CPA$_{ST3}$, or CPA$_{DAF}$ virus, and 48 h after infection, they were analyzed for cell surface expression of CPA by flow cytometry. Cells

Fig. 5. Retroviral infection with CPA$_{ST3}$ sensitizes cells to MTX-Phe in a dose-dependent manner. SCCVII cells were infected with LacZ or CPA$_{ST3}$ retrovirus and then treated with increasing doses of MTX or MTX-Phe for 48 h prior to plating for SF. CPA$_{ST3}$-infected cells treated with MTX (□), LacZ-infected cells treated with MTX-Phe (○), and CPA $_{ST3}$-infected cells treated with MTX-Phe (●) are shown. Bars, SE.

Table 2 Retroviral transduction sensitizes cells to MTX-Phe

SCCVII cells were infected with CPA$_{ST3}$ or LacZ retrovirus, treated with MTX or MTX-Phe for 48 h, and then assayed by colony formation. Data represent the mean ± SE of three experiments.

Drug (nM)	MTX	MTX-Phe	
		LacZ	CPA$_{ST3}$
IC$_{50}$	1.1 ± 0.2	>1000	2.5 ± 1.9
IC$_{95}$	25.3 ± 4.7	>1000	34.0 ± 5.3

A

PNKGSGTTSGTTRLLSGHTC FTLTGLLGTLVTMGLLT

membrane insertion

Phosphatidylinositol   cleavage
Linkage

Pro-Peptide | Catalytic Domain

B

Mock CPA$_{ST3}$ CPA$_{DAF}$ Mock CPA$_{ST3}$ CPA$_{DAF}$ Mock CPA$_{ST3}$ CPA$_{DAF}$ kDa

— 94
— 68
— 43
— 29
— 18

extracts   extracts   media
30 min   5 hrs

C

LacZ   CPA$_{ST3}$ CPA$_{DAF}$

Cell Number (all three)
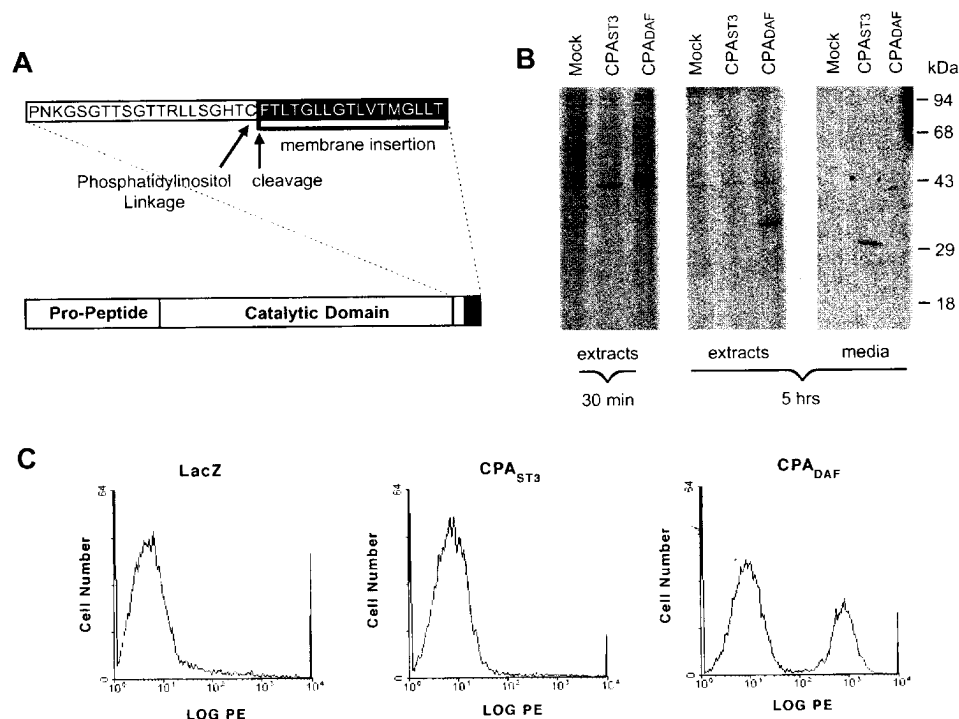
LOG PE (all three)

Fig. 6. Expression of CPA$_{DAF}$ results in cell surface-localized CPA without release into the conditioned medium. CPA$_{DAF}$ was constructed by fusing the 37-amino acid glycophospholipid membrane anchoring domain from DAF to the COOH-terminus of CPA (A). 293T cells were mock transfected or transfected with CPA$_{ST3}$ or CPA$_{DAF}$, and cell extracts or conditioned media were then analyzed by immunoprecipitation, SDS-PAGE, and autoradiography after a 30-min pulse with [^{35}S]cysteine/methionine or after a 30-min pulse, followed by a 5-h chase (B). SCCVII cells were infected with LacZ, CPA$_{ST3}$, or CPA$_{DAF}$ and then analyzed for cell surface expression of CPA by flow cytometry (C).

selection (400 μg/ml), and the number of G418-resistant colonies was used to calculate the "% infected cells" for each viral titer. In the second pair of dishes, cells infected at the same viral titers were treated with 1 μM MTX-Phe for 48 h, at which time they were plated and the SF subsequently calculated. These data were plotted as "surviving fraction" as a function of "% infected cells" (Fig. 7). In addition, to verify these infection rates, the cell surface construct was also assayed by flow cytometric analysis, which gave results consistent with G418 selection (data not shown).

When cultures were infected with varying dilutions of virus and then treated with MTX-Phe, the CPA$_{DAF}$-infected cultures were more sensitized to the drug than the CPA$_{ST3}$-infected cultures, even at equal rates of infectivity (Fig. 7). A culture of ~50% CPA$_{DAF}$-expressing cells exhibited a SF of <0.001 in the presence of 1 μM MTX-Phe, and the level of cytotoxicity decreased as the percentage of infected cells decreased to a SF of <0.1 at 5% CPA$_{DAF}$ expression. CPA$_{ST3}$, in contrast, peaked at a SF of slightly >0.001 for a 50% expressing culture, and there was little cytotoxicity seen below a 10% expressing culture (SF > 0.75).

CPA$_{DAF}$ Partially Protects from Collateral Cytotoxicity. Finally, to determine whether the release of secreted CPA$_{ST3}$ would sensitize cells distant from the site of production, a unique coculture assay was developed to measure the impact of the diffusion of CPA$_{ST3}$ on the cytotoxicity of nontransduced cells located some distance from the CPA-expressing cells. SCCVII cells that were 50% CPA$_{ST3}$ or 50% CPA$_{DAF}$ expressing were plated in the top chambers of a two-chamber, six-well tissue culture plate, and an equal number of nontransduced SCCVII cells were plated in the bottom chamber. The membrane dividing the two chambers had a 0.4 μm pore size, which was small enough to prohibit the passage of whole cells between the chambers; however, released CPA$_{ST3}$ and both MTX-Phe and MTX would readily diffuse between the chambers. The seeded cells were left in culture for 48 h, at which point MTX-Phe was added to the medium in both the top and bottom chambers to a final concentration of 1 μM, and the cells were plated for CFA at 12-h intervals.

Consistent with previous results, nontransduced parental cells exhibited no toxicity when exposed to the prodrug (Fig. 8). However, for CPA$_{ST3}$- and CPA$_{DAF}$-expressing cultures, cytotoxicity was observed for both the transduced wells (the top chamber) and the bystander cells (the bottom chamber). The toxicity appeared first in the top chambers and increased with the time of exposure to the prodrug. The level and rate of cytotoxicity in the top chambers was similar between CPA$_{ST3}$ and CPA$_{DAF}$ cultures throughout the course of the experiment (Fig. 8). However, there was a difference in collateral cytotoxicity to cells in the lower chambers, with cytotoxicity apparent earlier and to a greater extent in CPA$_{ST3}$ cultures than in CPA$_{DAF}$ cultures (Fig. 8). Therefore, the anchoring of CPA$_{DAF}$ to the surface of the cell conferred potent sensitization to both CPA$_{DAF}$-expressing and bystander cells in close proximity to each other (those in the top chamber) in a manner similar to CPA$_{ST3}$-expressing cells. Yet, although there was collateral cytotoxicity to cells some distance from the CPA$_{DAF}$-expressing cells (those in the bottom chamber), this cytotoxicity appeared later and to a lesser extent than that seen for CPA$_{ST3}$-expressing cells, where the enzyme could diffuse into the lower chamber and generate MTX *in situ*.

DISCUSSION

GDEPT strategies have been proposed as a means of achieving high intratumoral levels of chemotherapeutics with decreased systemic toxicity. The best studied of these include the HSV-TK/ganciclovir (20) and the CD/5-FC systems (21). One common feature of these systems is their reliance upon nonmammalian enzymes that therefore are highly immunogenic. Although the added immune response may contribute to the antitumoral effect (2), the destruction of transduced cells by the host immune system could also inhibit the efficacy of repetitive prodrug administration. The system detailed here is appealing in that it relies upon a mammalian enzyme that is highly conserved and therefore should be only mildly immunogenic; thus repetitive or long-term prodrug administration may be possible.

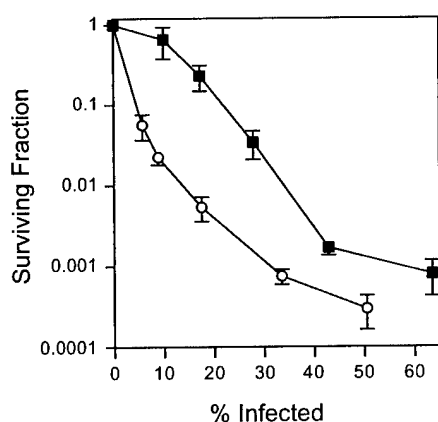In addition, for both the HSV-TK/ganciclovir and the CD/5-FC

Fig. 7. CPA$_{ST3}$- and CPA$_{DAF}$-expressing cells both exhibit a potent bystander effect. SCCVII cells were infected with increasing dilutions of CPA$_{ST3}$ or CPA$_{DAF}$ retrovirus and plated for colony formation in the absence or presence of G418 to determine percentage of infected cells. In parallel plates, CPA$_{ST3}$ (■) or CPA$_{DAF}$ (O) infected cells were treated with MTX-Phe for 48 h before plating for SF. Bars, SE.

trypsin-activated CPA. NH$_2$-terminal sequencing of endogenously activated CPA $_{ST3}$ also revealed that cleavage occurred at the expected location, liberating a mature peptide that is identical to the native mature protein. This is the first direct biochemical demonstration of the cleavage site for the ST3 linker region; the work that identified this cleavage domain was based upon site-directed mutagenesis to indirectly ascertain which site was cleaved (11).

CPA$_{ST3}$-transduced squamous cell carcinoma cells were able to generate MTX from MTX-Phe. Further evidence for the specificity of this activation was demonstrated by the inhibition of the conversion by the carboxypeptidase inhibitor derived from potatoes (data not shown). MTX was first detected 12 h after exposure to the prodrug, and by 72 h, >200 nM MTX was generated. Although this only amounted to a 20% conversion of MTX-Phe to MTX, the amount of MTX generated was still almost 10 times higher than the IC$_{95}$ of MTX in this cell line and thus was more than sufficient to cause potent cytotoxicity (see Table 2). These data are consistent with the notion that the MTX generated within the first 24 h inhibited cellular growth and the further production of CPA and, therefore, limited the final conversion of MTX-Phe to MTX. However, this MTX-mediated inhibition of further CPA production has not been proven.

Having ascertained that secreted active CPA$_{ST3}$ could indeed sensitize a number of different tumor cell lines to MTX-Phe after retroviral infection, we next developed a cell surface-associated form of endogenously active CPA. Unlike previous reports where carboxypeptidase G2 was still highly active both in vitro and in vivo when fused to the transmembrane domain of a cell surface receptor (32), we were unable to detect any functional CPA after construction of a similar fusion protein with CPA (data not shown). However, the use of the phospholipid membrane anchor from DAF resulted in a CPA molecule that not only remained cell tethered but was also functional, perhaps because of the increased conformational flexibility allowed by the lipid linkage as compared with a more rigid peptide transmembrane domain. This molecule, CPA$_{DAF}$, was able to sensitize SCCVII cells to MTX-Phe in a manner similar to the secreted form, and it appears to have retained the substrate specificity of the native molecule.

CPA$_{DAF}$ also sensitized SCCVII cells to MTX-Phe when only a very small fraction of cells (~5%) were expressing the protein. This may be attributable to the fact that time is not necessary for the molecule to build-up in the culture medium, because CPA$_{DAF}$ is anchored to the surface of the cell and does not diffuse away, and thus it is not removed when the culture medium is changed. The heightened cytotoxicity of CPA$_{DAF}$ when compared with CPA$_{ST3}$ also may be attributable to the local production of MTX at the cell surface, thus requiring lower total conversion levels of MTX-Phe to MTX to sensitize cells. This theory has been suggested for ADEPT protocols using CPA. Kuefner et al. (5) determined that when using antibody-conjugated CPA localized to the surface of the cell, 100-fold less enzyme was required to achieve equal sensitization as that found when purified CPA was simply added to the culture medium. They attributed this enhanced cytotoxicity to the production of MTX within

systems, it has been demonstrated that the "factory" or transduced cells are killed earlier and at lower doses of prodrug than nontransduced cells because of the intracellular accumulation of the toxic metabolites (22–26). In the case of HSV-TK/ganciclovir, this results in very little cytotoxicity when <50% of the cells in culture are expressing HSV-TK. Because of the fact that 5-fluorouracil is membrane permeable, there is a greater bystander effect for the CD/5-FC system. Unfortunately, we still have witnessed limited cytotoxicity with a low percentage of CD expressing cells both in culture (25) and in animal models (26). In the GDEPT reported here, extracellular production of MTX by soluble or cell surface CPA should equally sensitize both transduced and bystander cells with minimal preferential cytotoxicity.

One additional advantage of this system is that because MTX is one of the most widely used agents in the treatment of solid tumors, its pharmacokinetics, dose-limiting toxicities, and mechanisms of resistance are well understood (3, 4). HD-MTX therapy has been suggested as a means to circumvent tumor-derived resistance to MTX and is used quite commonly in current oncological practice. Recently, other strategies have been developed to genetically modify bone marrow stem cells to make them resistant to MTX so that HD-MTX treatment could be undertaken while biochemically protecting the bone marrow. These protective strategies have been demonstrated both for human bone marrow in culture (27) and for the protection of mice from MTX toxicity after transplantation of MTX-resistant bone marrow (28). Although these strategies have provided promising results, they do not offer any protection to the gastrointestinal tract, which is also highly sensitive to MTX-induced toxicity. The GDEPT proposed here by localizing HD-MTX to the tumor site may be able to increase the cytotoxic dose delivered to the tumor while protecting both the gastrointestinal tract and the bone marrow. An additional advantage for the use of CPA in an enzyme/prodrug strategy is that other antifolates, which have proven cytotoxic even in MTX-resistant cell lines (29), also rely upon transport through the reduced folate carrier. Therefore, these drugs could be converted to α-peptide blocked prodrugs to be used in this enzyme/prodrug strategy (30, 31).

To render CPA active in the absence of trypsin-dependent zymogen cleavage, the 10-amino acid linker region from ST3 was incorporated into CPA between the pro- and mature domains such that if cleavage occurred at the expected site, the mature peptide released would be identical to trypsin-activated CPA. Enzymatic analysis of purified CPA$_{ST3}$ confirmed the correct activation of the zymogen to the catalytic form for its kinetic profile was indistinguishable from the

Table 3 Retroviral transduction with CPA$_{ST3}$ or CPA$_{DAF}$ sensitizes cells to MTX-α-peptide prodrugs

The IC$_{50}$s of five MTX-α-peptide prodrugs were evaluated using a 96-well plate growth inhibition assay after retroviral transduction of SCCVII cells with LacZ, CPA$_{ST3}$, or CPA$_{DAF}$ retrovirus. Data represent the average of at least eight replicate wells.

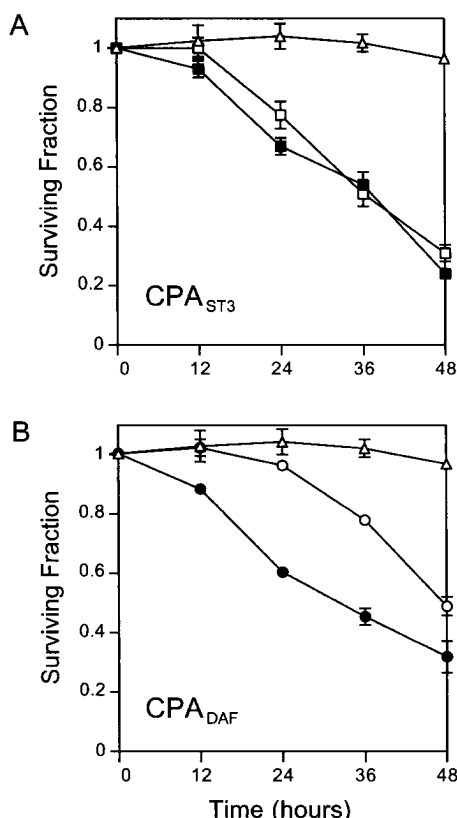| MTX-α-peptide	LacZ	CPA$_{ST3}$	CPA$_{DAF}$
Trp	4000	4000	4000
Gln	3000	2000	3000
Met	6000	2000	3000
Tyr	5000	450	1000
Phe	15,000	200	500

663

Fig. 8. CPA_{DAF} expression sensitizes infected and "bystander cells" to MTX-Phe while exhibiting reduced collateral toxicity. CPA_{ST3}- or CPA_{DAF}-expressing SCCVII cells were mixed at a 50:50 ratio with parental SCCVII cells and plated in the top of a dual chamber tissue culture plate. In the bottom chamber, an equal number of parental SCCVII cells were plated. Both chambers were then treated with 1 μM MTX-Phe, and parallel wells were plated for SF at 12-h intervals. Parental cells alone exposed to MTX-Phe ($\triangle$), cells from the top culture ($\blacksquare$, $\bullet$), or cells from the bottom culture ($\square$, $\bigcirc$) are shown. A, CPA_{ST3} cocultures ($\square$, $\blacksquare$). Bars, SE. B, CPA_{DAF} cocultures ($\bigcirc$, $\bullet$). Bars, SE.

the microenvironment around cell surface-localized CPA-antibody conjugates. In addition, by prohibiting the diffusion of the catalytically active enzyme away from transduced cells, CPA_{DAF} also partially inhibited collateral toxicity to cells more distant from the site of enzyme production than that seen for CPA_{ST3}, a fact that may be more readily apparent and more critical when this strategy is evaluated *in vivo*.

The studies reported here have focused on MTX-Phe, because it was identified as the best substrate for wild-type CPA (17). However, it has recently been demonstrated that this compound, unlike predictions, is not stable *in vivo* for it is rapidly converted to MTX after injection into mice, which prohibits direct evaluation of this GDEPT in an animal model (8). To overcome the unsuitability of MTX-Phe, modified MTX-α-peptide prodrugs with nonnatural amino acid blocking groups have been described that are poor substrates for endogenous systemic CPA-like activities and are thus highly stable *in vivo*. For example, the MTX-3-cyclopentyl-Tyr prodrug is 50,000-fold more stable in the presence of wild-type CPA than MTX-Phe (8). In addition, although these compounds are poor substrates for wild-type CPA, they are efficiently cleaved by the T268G mutant of CPA, which has an alteration in the substrate binding pocket. The efficacy of the endogenously active soluble (CPA_{ST3}) and cell surface forms of CPA (CPA_{DAF}) when combined with the T268G altered specificity form of the enzyme are now being evaluated. Preliminary evidence, which is in accordance with Smith *et al.* (8), suggests that expression of this T268G mutant in culture confers sensitization to MTX-3-

cyclopentyl-Tyr, whereas expression of the wild-type enzyme has no such capacity.

Recently, studies using the T268G mutant of human CPA in an ADEPT protocol were unable to demonstrate a clinical response because of the rapid inactivation of CPA *in vivo* (31). To allow time for distribution, binding, and subsequent clearance of unbound CPA/antibody conjugates, the authors waited 24 h after injection before they initiated prodrug treatment. However, the half-life of the enzyme/antibody conjugate *in vivo* was found to be significantly less than this, and as a result, there was little conversion of MTX-prodrugs. The CPA GDEPT strategy described here may circumvent this limitation through the continuous production of CPA by virally transduced cells, thus limiting the impact of protein inactivation. Because of the clinical efficacy of MTX in the treatment of squamous cell cancer of the head and neck and to the difficulty in achieving local control of head and neck cancer (33), future studies will be focused on the use of this GDEPT in the treatment of head and neck cancer by direct injection of CPA_{ST3}- or CPA_{DAF}-expressing adenoviruses into submental tumors in an animal model of head and neck cancer (26, 34, 35).

ACKNOWLEDGMENTS

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Combined Radiation and Enzyme/Prodrug Treatment for Head and Neck Cancer in an Orthotopic Animal Model

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In an effort to improve the therapeutic outcome for squamous cell cancer of the head and neck, we have used the enzyme cytosine deaminase (CD) and the prodrug 5-fluorocytosine (5-FC) as a means to deliver the chemotherapeutic agent 5-fluorouracil (5-FU) in a tumor-specific manner and have evaluated the use of this treatment in combination with external-beam radiation. Infection of SCCVII cells in culture with a CD-expressing retrovirus and treatment with 5-FC was cytotoxic depending on the time of treatment and dose of 5-FC. An orthotopic model of squamous cell cancer of the head and neck was used *in vivo* to study the CD/5-FC system both alone and with concurrent radiation due to the radiosensitizing properties that 5-FU generates *in situ*. Treated mice were imaged using magnetic resonance imaging (MRI), and their survival was evaluated. Neither 5-FU nor radiation either alone or combined provided a survival advantage. In contrast, 5-FC treatment prolonged survival and decreased tumor burden compared to control animals, but the tumors recurred after the treatment ceased. Finally, combined treatment with concurrent administration of 5-FC and radiation resulted in a synergistic decrease in tumor growth and enhanced survival over treatment with 5-FC or radiation alone. © 1999 by Radiation Research Society

INTRODUCTION

Squamous cell carcinoma of the head and neck is a common malignancy, with approximately 50,000 cases annually in the United States (*1*). Two-thirds of patients present with stage III or IV disease, for which traditional management consists of surgical resection followed by postoperative radiotherapy or, for patients with unresectable disease, chemotherapy and radiotherapy (*2*). The results of such treatment are unsatisfactory, with 5-year survival rates for stage III and IV disease of less than 25%. In addition, since only

about 20% of patients with squamous cell carcinoma of the head and neck develop metastatic disease, an increase in local control would most likely translate into a survival advantage (*1*). Attempts to intensify nonsurgical therapy are hindered by normal-tissue toxicity, specifically local mucosal reactions and systemic toxicity from chemotherapy (*3*). A promising treatment approach for patients with locally advanced squamous cell carcinoma of the head and neck has been to administer concurrent chemotherapy and radiotherapy; however, the toxicities of the combined treatments are often prohibitively great, thus necessitating a reduction in the intensity of either or both treatment modalities (*4*). In an attempt to circumvent some of these toxicities, gene-dependent enzyme prodrug therapies have been developed (*5*). In these strategies, tumors are genetically modified with an enzyme that is not present or is present at low levels in normal tissues. Subsequently, a prodrug is administered systemically, which is nontoxic except when the prodrug is metabolized *in situ* by the enzyme expressed within the modified tumor cells. Thus, in principle, localized and higher concentrations of an active agent can be achieved within the tumor milieu, thereby minimizing systemic toxicities.

5-Fluorouracil (5-FU) is one of the most active agents used in the treatment of squamous cell cancer of the head and neck both as part of combined chemotherapy protocols and with concomitant radiotherapy, where it has been shown to be a potent radiosensitizer (*6*). 5-Fluorocytosine (5-FC) is a nontoxic prodrug of 5-FU that can be converted by the bacterial enzyme cytosine deaminase (CD) into 5-FU (*7*). The combination of CD/5-FC as a gene-dependent enzyme prodrug treatment has been studied both alone and in combination with radiation (*8–11*), and an improved response was observed with the combination compared to either treatment alone. These studies, however, have focused on hind-limb (*10*) or flank tumors (*11*) grown as xenographs in nude mice. Given that the dose-limiting toxicities for the combination of radiation and 5-FU in the treatment of squamous cell carcinoma of the head and neck are typically mucositis and local-regional toxicities, the studies of the CD/5-FC treatment protocol in flank tumors may not provide an accurate evaluation of the utility of this

system. In this study, we have evaluated the combination of the CD/5-FC enzyme prodrug strategy with or without concomitant radiation in an immune-competent orthotopic model of squamous cell carcinoma of the head and neck (*12*). Since this model more closely parallels the natural history and clinical treatment of squamous cell carcinoma of the head and neck, it may provide valuable insight into the clinical utility of the CD/5-FC treatment protocol, both alone and in conjunction with external-beam radiation.

MATERIALS AND METHODS

Cell Culture

SCCVII cells, a murine squamous cell carcinoma line, and ΦXa retroviral producer cells (kindly provided by Gary Nolan, Stanford, CA) were cultured under standard conditions in DMEM supplemented with 10% FBS, penicillin, streptomycin and L-glutamine.

Production of Retrovirus

The isolation and expression of the *E. coli CD* gene has been described previously (*13*). The *CD* expression plasmid from this work was digested with *Eco*RI to isolate the *CD* cDNA along with an added Kozack sequence, and this fragment was subcloned into Lzr.pBMN (kindly provided by Gary Nolan, Stanford, CA) to generate Lzr.CD. ΦXa retroviral producer cells in 100-mm tissue culture dishes were transfected with 15 μg of the Lzr.LacZ or Laz.CD retroviral plasmids using calcium phosphate precipitation, and retroviral supernatants were harvested 72 h later, pooled, divided into aliquots, and stored at −70°C. SCCVII cells were infected using these retroviral supernatants supplemented with polybrene (16 μg/ml, Sigma, St. Louis, MO).

Cytotoxicity Assays

Cytotoxicity assays were performed using a standard assay of colony-forming ability (*14*). Cells were plated in 60-mm tissue culture dishes at a density of 2000 cells/cm², and 18–24 h later they were infected with retroviral supernatants for 18–24 h, at which time the medium was changed to one supplemented with PBS or 5-FC (Sigma). Cells were treated with the drug for 0–72 h, at which point they were plated for colony formation, and after 7–10 days the dishes were fixed and stained with crystal violet before counting. The data plotted represent the mean and standard error of at least three experiments.

Stable Cell Lines

Stable CD-expressing SCCVII cell lines were established by retroviral infection and limiting dilution in 96-well plates. Twenty clones were isolated, and the best-éxpressing clones were verified by cytotoxicity assays and western blotting. A CD western blot was performed using the protocol described previously (*15*) using a rabbit α-CD antibody [1:5000 dilution, provided by T. S. Lawrence, University of Michigan (*16*)] followed by enhanced chemiluminescence (Pierce, Arlington Heights, IL).

In Vivo Model

Animal experiments, including designations for survival outcomes, were approved by the University of Michigan Committee on Use and Care of Animals. A modified form of an immune-competent model of squamous cell carcinoma of the head and neck was used (*17*). Briefly, CD-expressing or parental SCCVII cells growing in culture were trypsinized, washed in PBS, counted, and resuspended at a concentration of 4×10^7 cells per milliliter in PBS. Fifty microliters of this cell solution, containing 2×10^6 cells, was then injected into the submental space of C3H mice (6–8 weeks, Charles River Laboratories, Inc., Wilmington, MA) along the midline using an external approach (*18*).The presence of

tumors was verified by MRI 3–4 days after injection. Treatment with saline, 5-FC (500 mg/kg daily, i.p.) or 5-FU (25 mg/kg daily, i.p.) was initiated on day 5 after implantation of tumor cells and continued for a total of 10 days.

Irradiation of Tumors

Tumors were irradiated as described previously (*19*) using a Theratron 80 ^{60}Co γ-ray source at a dose rate of 1.1 Gy per minute and a source-to-skin distance of 80 cm. Radiation was delivered in five consecutive daily fractions starting 5 days after tumor implantation. For combined treatment, 5-FC or 5-FU was administered daily for 5 days with the drug given in the morning and radiation in the afternoon; the drug was then given for an additional 5 days without radiation. Mice were anesthetized with pentobarbital (60–70 mg/kg, i.p.) and then placed in a custom-designed apparatus in the lateral decubitous position in a radial arrangement (snouts at the center). To deliver the full dose to the tumor, a 0.5-cm layer of bolus was placed to compensate for build-up. The tumors of all five mice were irradiated simultaneously in a single field, and the remaining parts of their bodies were shielded by a cerrobend block 7 cm thick. Thermoluminescent dosimeters placed under the cerrobend measured 3% of the dose at the central ray of the treatment field. In addition, individual $2 \times 2 \times 7$-cm cerrobend blocks were placed by hand on a Plexiglas platform to shield the cranium and eyes of each mouse. Mice were weighed every 2–3 days and euthanized if they had lost >30% of their body weight. The mean time of survival ± the standard error of the mean after tumor implantation were calculated for each treatment group, and the statistical significance for survival between treatment groups was evaluated using Student's *t* test for unpaired data.

Evaluation of Tumor Growth by MRI

Growth of tumors during and after treatment was monitored using sequential MRI studies over 3 weeks on a Varian MR system (Varian Instruments, Freemont, CA) equipped with a 7-T, 18.3-cm horizontal bore magnet (Oxford Instruments, Oxford, UK) with actively shielded gradients. In brief, for MRI examination, mice were anesthetized with sodium pentobarbital (60–70 mg/kg, i.p.) and positioned within a small radiofrequency quadrature coil (USA Instruments, Aurora, OH). A single-slice gradient-recalled echo image was acquired with 1-mm "saturation crosshairs" imprinted on the axial and transverse images to facilitate rapid and reproducible positioning on the animal. Multislice coronal T2-weighted images were acquired by using a spin echo sequence with the following parameters: 3.5 s repetition time, 60 ms echo time, field of view = 3×3 cm using a 128×256 matrix, slice thickness = 1.0 mm, slice separation = 0 mm, number of slices = 24, and four signal averages per phase encode step.

In vivo SCCVII tumor volumes were quantified from the multislice MR images by outlining the region of interest electronically using image-processing software (Advanced Visual Systems, Waltham, MA) as described previously (*20*). The number of pixels was converted to a volume, and the total tumor volume was calculated by summing the tumor volumes from all slices. Tumor volumes obtained over time were used to calculate tumor growth rates for individual animals.

RESULTS

Retroviral Transduction with CD Sensitizes SCCVII Cells to 5-FC

To evaluate the efficacy of CD/5-FC for treatment of head and neck cancer, an amphatrophic retroviral vector expressing CD under the control of the Moloney murine leukemia virus long terminal repeat was developed. Retroviral stocks for both control (β-galactosidase expressing) and CD-expressing virus were prepared in parallel under
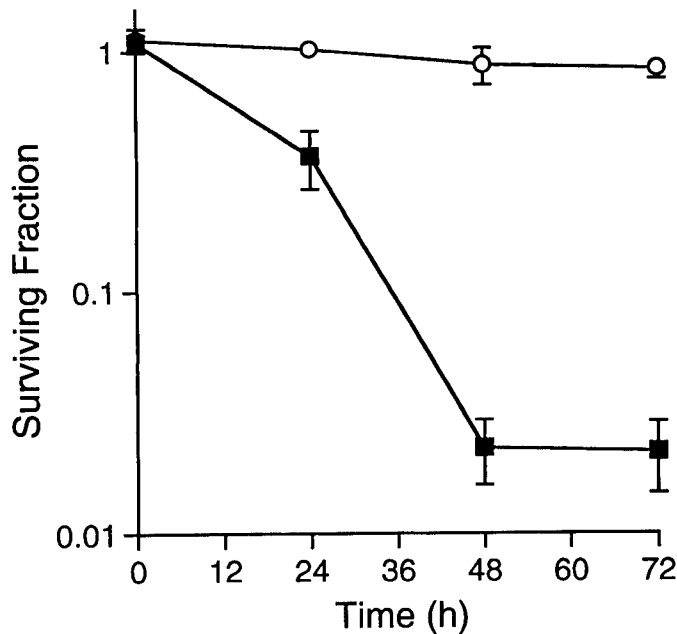
FIG. 1. CD expression sensitizes SCCVII cells to 5-FC. SCCVII cells were infected with LacZ (open circles) or CD-expressing (closed squares) retroviruses and treated with 100 μM 5-FC for 0–72 h before plating for assay of colony-forming ability. Data represent the mean ± the standard error of three experiments.

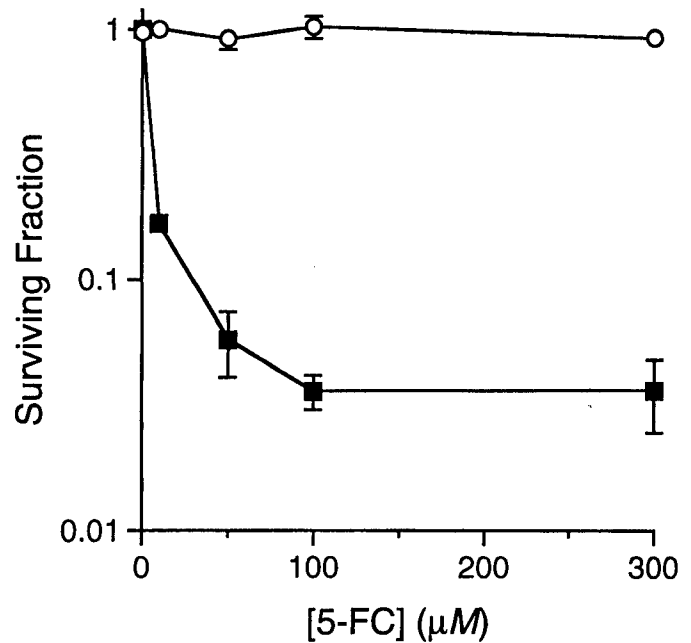**FIG. 2.** CD expression sensitizes SCCVII cells to 5-FC in a dose-dependent manner. SCCVII cells were infected with LacZ (open circles) or CD-expressing (closed squares) retroviruses and treated with 0–300 μM 5-FC for 48 h before plating for assay of colony-forming ability. Data represent the mean ± the standard error of three experiments.

identical conditions, and a single large batch for each preparation was divided into aliquots, frozen, and used throughout the experiments. Infection of SCCVII cells with this LacZ virus resulted in a 50–60% infection rate as determined by X-gal staining (data not shown).

To evaluate the ability of CD expression to sensitize SCCVII cells to 5-FC, cells in culture were infected with the CD-expressing retrovirus or the control LacZ virus, treated with 100 μM 5-FC, and plated to determine the surviving fraction (SF) at intervals from 0 to 72 h (Fig. 1). There was no apparent cytotoxicity to SCCVII cells infected with the LacZ retrovirus and exposed to 5-FC for 72 h (SF > 0.9). The cells infected with CD-expressing retrovirus, however, were sensitized to 5-FC in a time-dependent manner with a plateau after 48 h at an SF of <0.04.

To study the dose-dependent nature of the sensitization by 5-FC, SCCVII cells were infected with LacZ- or CD-expressing retroviruses and treated with increasing concentrations of 5-FC for 48 h, at which time they were plated to determine their colony-forming ability (Fig. 2). There was no apparent cytotoxicity to cells infected with LacZ-expressing retrovirus when they were treated with up to 300 μM 5-FC (SF > 0.9), and an IC_{50} value could not be determined (Fig. 2). However, SCCVII cells infected with CD-expressing retrovirus were sensitized to 5-FC in a dose-dependent manner, with IC_{50} and IC_{95} values of 6 and 72 μM, respectively (Fig. 2). Maximal cytotoxicity (SF < 0.03) was observed at 100 μM 5-FC, with no increase in cytotoxicity with increasing concentrations of 5-FC beyond 100 μM 5-FC.

Generation of Stable CD-Expressing SCCVII Cells and Evaluation of Tumor Growth In Vivo

The response to any gene-dependent enzyme prodrug treatment *in vivo* will depend on the percentage of tumor cells expressing the transgene. Therefore, to closely control this variable, a stable SCCVII cell line expressing CD was developed. Western blots of LacZ and CD-expressing SCCVII cells indicated that the *CD*-transduced clone expressed CD, as seen by the band at approximately 48 kDa that was not present in LacZ-infected SCCVII cells (Fig. 3). In addition, cytotoxicity assays indicated that this cell
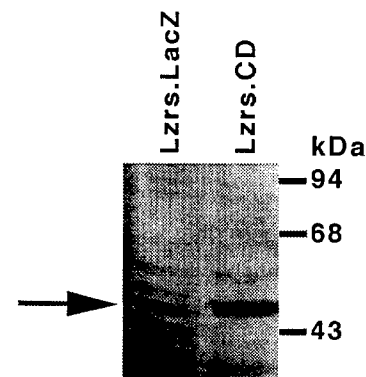

FIG. 3. Retroviral transduction with *CD* results in expression of CD protein. SCCVII cells were infected with LacZ or CD-expressing retroviruses and individual clones were isolated and analyzed by SDS-PAGE and western blotting. The location of the molecular weight marker is indicated on the right, and the band representing CD is indicated with an arrow.
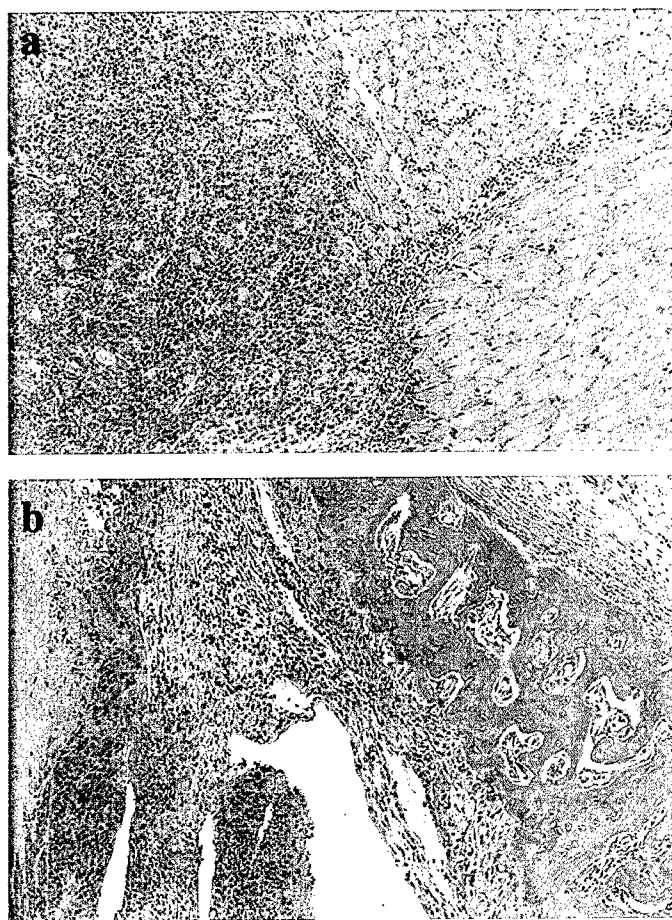
FIG. 4. Microscopic examination of tumor specimens growing in the submental region of C3H mice. Hematoxylin and eosin staining of tumor sections revealed poorly differentiated squamous cell carcinoma with many mitotic figures that was invasive into normal structures. Panel A shows invasion of musculature (right) by squamous cell carcinoma (left). Panel B demonstrates encroachment on bone (right) by squamous cell carcinoma (left).

FIG. 5. CD-expressing tumors exhibited growth inhibition after 5-FC treatment *in vivo.* Representative T2-weighted coronal MR images through the largest cross-section of the tumors are displayed for parental SCCVII tumors that were treated with saline (a) or for CD-expressing tumors that were treated with 5-FU (b) or 5-FC (c). Images for each treatment group were acquired from the same mouse on days 5, 9 and 14 after implantation. The dimensions of the tumors are indicated with arrows for the image taken 14 days after implantation.

line had a sensitivity to 5-FC that was similar to that of pooled retrovirally transduced populations (data not shown).

Submental tumors were established in C3H mice using CD-expressing or nonexpressing SCCVII cells. The presence of tumors was established by MRI 3–4 days after implantation, and the tumors grew rapidly thereafter, with a doubling time of less than 28 h (see Figs. 5A and 6A). The tumor and submental region from two untreated animals were excised surgically and submitted for pathological sectioning and staining (Fig. 4). This analysis revealed a poorly differentiated squamous cell carcinoma with many mitotic figures that at an earlier time (10 days after implantation) was highly invasive into the floor of the musculature of the mouth (Fig. 4A). At the later time (15 days after implantation), the tumor invaded other soft tissues and encroached on bone within the head and neck (Fig. 4B). These results are consistent with previous reports using this animal model (*12, 17*).
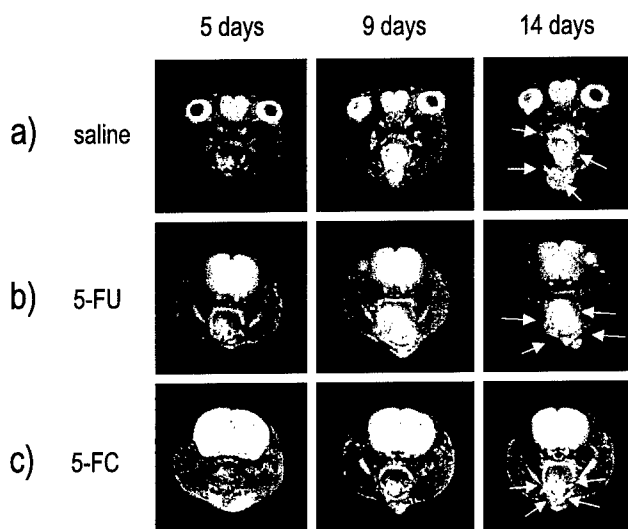
5-FC Treatment of CD-Expressing SCCVII Cells Results in Decreased Tumor Growth Rate and Increased Survival

Treatment was initiated on day 5 after tumor implantation when the presence of tumors had been confirmed by MRI. The tumors were of similar size at the start of the study with a mean tumor volume of 11.7 ± 0.9 mm^3. To monitor the extent and rate of tumor growth *in vivo*, one mouse from each group was selected randomly to be followed by sequential MRI both during and after treatment. Representative MR images through the largest cross section of tumors on days 5, 9 and 14 after tumor implantation are shown for mice treated with saline, 5-FU and 5-FC (Fig. 5A–C). The MR images shown represent single 1-mm slices through the heads of individual mice. Depending on the position of the mouse and the position of the tumor within the submental region of the mouse, different structures of the head are visible, including the optic lobe (Fig. 5A) and the brain (Fig. 5B and C). SCCVII tumors are clearly delineated, without the need for a contrast agent for enhancement, as a hyperintense lesion in the lower part of the image and have been outlined using arrows in the last images in each of the three series (Fig. 5A–C).

Relative tumor volume as a function of days after implantation was plotted based on the volumes determined from the sequential MR studies (Fig. 6A). Untreated parental or CD-expressing tumors had similar growth rates with doubling times of 1.0 and 1.3 days, respectively (Figs. 6A and 7A). Treatment with 5-FU at the maximum tolerated dose (25 mg/kg i.p. daily for 10 days) had little impact on tumor doubling time, which was 1.4 days. As predicted from the *in vitro* analysis, treatment of tumors derived from
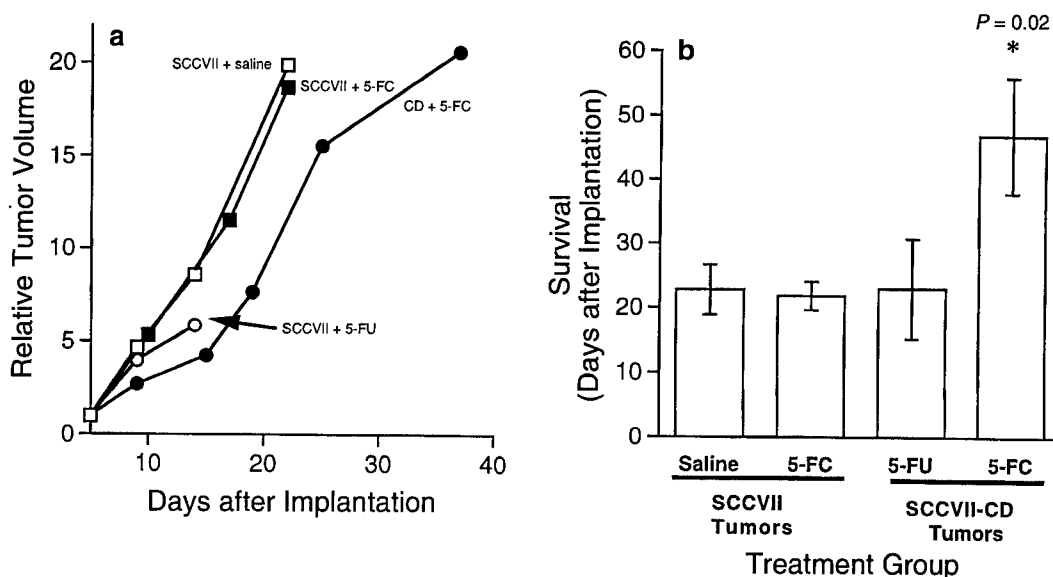
FIG. 6. 5-FC treatment results in a tumor growth delay and survival advantage for mice bearing CD-expressing tumors. Panel A: Relative tumor volume as a function of days after implantation is plotted based on volumetric data acquired from MRI images. Animals were treated with 10 daily doses of saline, 5-FU (25 mg/kg) or 5-FC (500 mg/ kg). Parental SCCVII cells were treated with saline (open squares) or 5-FC (closed squares), and CD-expressing tumors were treated with 5-FU (open circles) or 5-FC (closed circles). Panel B: Mean survival for the treatment groups ± the standard error, $n = 5$ for all groups. Statistically significant increases in survival compared to saline-treated controls are indicated with an asterisk (*), and the P values are given.

parental SCCVII cells with 5-FC (500 mg/kg, i.p. daily 10 days) also had no impact on tumor growth, with an observed tumor doubling time of 1.1 days. CD-expressing tumors, however, had a decrease in tumor growth after the initiation of 5-FC treatment, with a tumor doubling time

2.4 times longer than that of untreated tumors. Upon termination of 5-FC treatment, tumor growth promptly returned to a rapid rate.

In addition to MRI studies, the validity of this gene-dependent enzyme prodrug treatment was also evaluated by
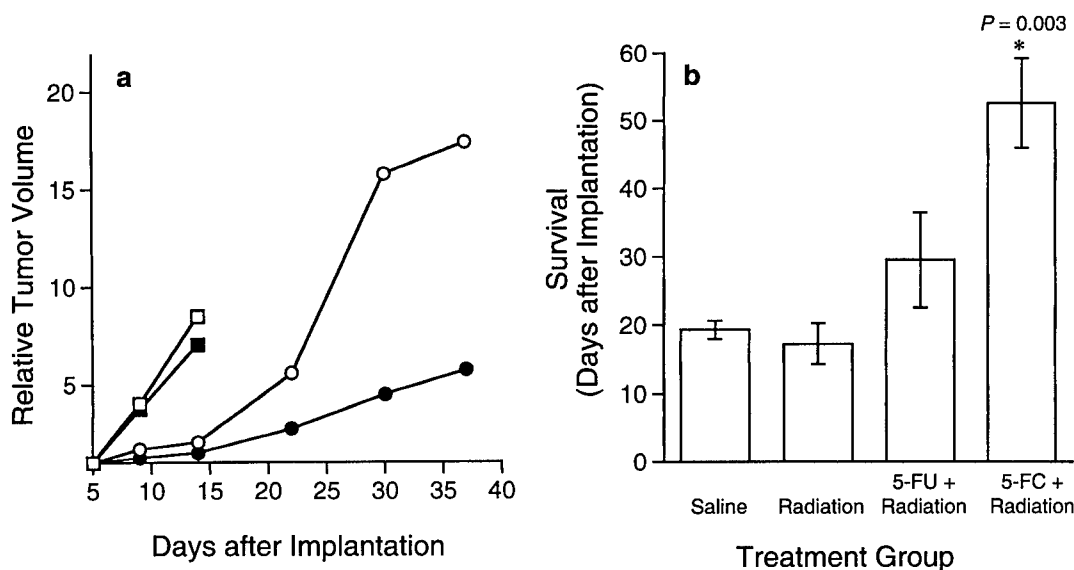

FIG. 7. Combined enzyme prodrug treatment and radiation increase tumor response and survival. Panel A: Tumor volumes were measured for CD-expressing SCCVII tumors as described for Fig. 6 except that mice were treated with 5 daily fractions of 4 Gy (as described in the Materials and Methods) in conjunction with a 10-day course of saline, 5-FU or 5-FC. Saline-treated control (open squares), saline + radiation (closed squares), 5-FU + radiation (open circles), and 5-FC + radiation (closed circles). Panel B: Mean survival for the treatment groups ± the standard error, $n = 5$ for all groups except for radiation alone, where $n = 4$. Statistically significant increases in survival compared to saline-treated controls are indicated with an asterisk (*), and the P values are given.

monitoring the survival of mice bearing parental or CD-expressing SCCVII tumors (Fig. 6B). The mean survival time of saline-treated tumor-bearing animals was 22.8 ± 4 days, and there was no increase in survival for treatment of mice bearing tumors derived from parental SCCVII cells with 5-FC, with a survival time of 20.2 ± 1.8 days. Similarly, after treatment with 5-FU, animals bearing CD-expressing tumors had a mean survival of 23.0 ± 7.8 days, which also was not prolonged compared to that of saline-treated controls. In contrast, there was an almost twofold increase in survival to 46.8 ± 9.0 days for animals bearing CD-expressing SCCVII tumors that were treated with 5-FC ($P < 0.02$).

Radiation in Conjunction with CD/5-FC Results in Both Increased Inhibition of Tumor Growth and Increased Survival

The first *in vivo* experiments established that treatment with 5-FC was more beneficial than treatment with 5-FU as assessed by MRI studies and survival of animals. Therefore, we next evaluated whether CD/5-FC in combination with concurrent radiation was superior to fractionated radiation with or without 5-FU. Animals were implanted with CD-expressing SCCVII cells, and drug treatment with or without radiation was initiated 5 days after tumor cell implantation for animals with tumors as confirmed by MRI (mean volume 6.5 ± 1.0 mm³). 5-FC and 5-FU were administered using the same schedule as in the previous experiment. In addition, tumor-bearing animals were also treated with fractionated radiation (4 Gy per day for 5 days starting 5 days after tumor implantation, as described in the Materials and Methods) either alone or in combination with concurrent 5-FC or 5-FU treatment. As in the previous experiment, the tumor growth *in vivo* both during and after treatment was measured by MRI (Fig. 7A), and the survival of the treatment groups was monitored (Fig. 7B).

Radiation alone had little impact on the rate of tumor growth, with a doubling time of 1.5 days that was similar to that of the tumors in the saline-treated animals, which had a doubling time of 1.3 days (Fig. 7A). Although neither 5-FU nor radiation alone had a pronounced effect on tumor growth rates, the combination of these two treatments was synergistic, with an increase in tumor doubling time from 1.3 to 8.2 days. Similarly, the addition of radiation to 5-FC in the treatment of CD-expressing tumor cells also synergistically increased the tumor doubling time to 9.2 days. However, despite a significant growth inhibition during treatment, the tumors treated with 5-FU and radiation resumed rapid growth at the end of treatment. In contrast, the growth of the tumors treated with 5-FC and radiation was suppressed to a greater extent and for a longer time even after treatment ceased (Fig. 7A).

The survival of the different treatment groups (Fig. 7B) paralleled the results observed for the *in vivo* tumor growth rates determined by MRI (Fig. 7A). Fractionated radiation

alone offered no survival advantage when compared to saline-treated controls, with mean survival times of 17.3 ± 3.0 and 19.3 ± 1.3 days, respectively. Although there was an increase in the time of survival for the group treated with 5-FU and radiation to 29.5 ± 7 days, this increase was not statistically significant ($P > 0.1$). In contrast, the time of survival for the group treated with 5-FC and radiation was extended more than 2.5-fold compared to that of the saline-treated animals to 55.0 ± 14 days ($P < 0.025$). Thus, although both 5-FU/radiation and 5-FC/radiation showed impressive inhibition of tumor growth, only the growth inhibition by 5-FC/radiation was long-term and translated into a survival advantage.

CD/5-FC in Combination with Radiation Results in Inhibition of Tumor Growth and Enhanced Survival Even When Less Than 100% of the Tumor Is CD-Expressing

The clinical applicability of any gene therapy strategy for cancer will depend on an antitumor response even when significantly less than 100% of the cells in the tumor are expressing the gene of interest. Therefore, we performed *in vivo* mixing experiments where tumors were established by combining 0, 10, 25, 50 or 100% CD-expressing cells with parental SCCVII cells at the time of implantation. Treatment with 5-FC daily for 10 days and concurrent irradiation for 5 days was initiated starting 5 days after implantation, when tumors had been confirmed by MRI (mean volume 11.5 ± 1.4 mm³). After the initiation of treatment with 5-FC and radiation, there was a decrease in the rate of tumor growth for all CD-expressing tumors compared to saline-treated controls and to parental SCCVII tumors (Fig. 8A), and the magnitude of this lag was directly dependent on the percentage of CD-expressing cells. For low percentages of CD-expressing cells, the growth delay was small, with only a 2.2-fold growth rate for the tumor established with 10% CD-expressing cells. However, the rate of growth increased to 4.0-, 7.3- and 16.5-fold for tumors derived from 25, 50 and 100% CD-expressing cells, respectively.

In addition, there was a trend toward increased survival as a function of increasing percentage of CD-expressing cells (Fig. 8B). Saline-treated control animals had a mean survival of 21.9 ± 2.2 days that was effectively unchanged for parental SCCVII tumors treated with 5-FC and radiation (23.5 ± 4 days). The inclusion of as little as 10% CD-expressing cells increased survival to 25.3 ± 2.6 days, but this increase, although suggestive, was not statistically significant ($P > 1.0$). However, the expression of CD by 25% of the tumor cells increased the time of survival after treatment with 5-FC and radiation to 30.5 ± 1.6 days ($P < 0.02$). This was further increased to 39.5 ± 6.1 and 50.0 ± 1.1 days for 50% ($P < 0.003$) and 100% ($P < 0.0004$) CD-expressing cells, respectively.

DISCUSSION

Because local regional control is paramount in the treatment of squamous cell carcinoma of the head and neck (2),
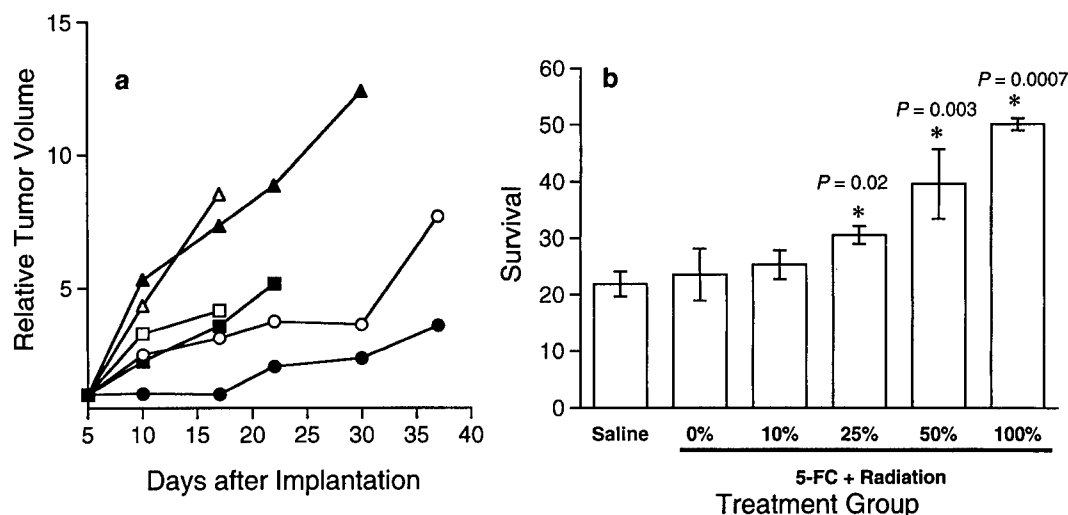
FIG. 8. Tumor growth inhibition and increased survival after combined treatment with 5-FC and radiation in mixed CD-expressing and nonexpressing tumors. Panel A: Tumor volumes were measured as described in Fig. 6 for tumors composed of 0, 10, 25, 50 or 100% CD-expressing cells. All animals were treated with a combination of 5-FC and radiation except for saline-treated controls (open triangles). 0% CD-expressing (closed triangles), 10% CD-expressing (open squares), 25% CD-expressing (closed squares), 50% CD-expressing (open circles), and 100% CD-expressing (closed circles). Panel B: Mean survival for the treatment groups ± the standard error; $n = 4$ for all groups except saline-treated control, where $n = 6$. Statistically significant increases in survival compared to saline-treated controls are indicated with an asterisk (*), and the P values are given.

using a system to impart more aggressive local treatment and diminish regional and systemic toxicity theoretically should increase both local control and survival. The combination of 5-FU and radiation has been used in the treatment of squamous cell carcinoma of the head and neck, and it has typically resulted in an increased clinical response but infrequently in enhanced survival (6). The CD/5-FC gene-dependent enzyme prodrug treatment either alone or in conjunction with radiation is therefore a logical choice for treating this tumor, since local intensification in chemotherapy may decrease the dose-limiting toxicities encountered in traditional therapy of the tumor while also resulting in an enhanced clinical response.

In vitro SCCVII cells were sensitized to 5-FC in a time- and dose-dependent manner after infection with a CD-expressing retrovirus. Since the in vitro analysis was promising, we next established a stable CD-expressing SCCVII cell line to study this treatment strategy in vivo. The use of a stable cell line, while not necessarily paralleling a clinical gene therapy protocol, enabled us to accurately control the percentage of CD-expressing cells within the tumor population to determine the threshold of expressing cells that would be necessary to achieve a clinical benefit.

The immune-competent orthotopic model for small cell carcinoma of the head and neck that we used was described previously for use in the treatment of head and neck cancer with the HSV-TK/ganciclovir enzyme/prodrug system (12), but it has not been studied for other gene-dependent enzyme prodrug treatment strategies. An advantage of this model when compared to flank tumor models is that the tumor growth and invasion into the submental musculature

and the oral cavity after orthotopic implantation directly parallel the course of squamous cell carcinoma of the head and neck seen in humans. In addition, both regional lymph node and pulmonary metastasis have been described after submental injection (12, 17, 18), a phenotype that is only rarely observed in flank tumor xenographs. Also, the toxicities of both the disease and the treatment in this model would be expected to parallel those witnessed when treating squamous cell carcinoma of the head and neck, and thus this model may provide a better way to evaluate the efficacy of treatment than a flank tumor model (12, 17, 18). Previous reports on this orthotopic model, however, have relied on surgical resection and measurement of tumors both shortly after implantation and at the end of the experiment to quantify tumor burden. Our MRI data indicate that this tumor has a profoundly endophytic component, which would make accurate evaluation by surgical dissection both technically difficult and time-consuming. Further, MRI offers the advantage of being able to noninvasively monitor tumor growth dynamically both during and after treatment (20). Indeed, the growth rates that we determined by MRI were consistent with and predictive of the final results that we obtained for animal survival. The obvious advantage of the MRI studies is that the results are obtained in days or weeks instead of a month or more. In addition, since the MRI-based evaluation is noninvasive, it is possible to perform these studies concurrent with ongoing survival experiments.

Treatment of animals bearing 100% CD-expressing tumors with 5-FC resulted in a greater than twofold decrease in the rate of in vivo tumor growth compared to saline-treated control animals, and this translated directly into a

twofold increase in time of survival. 5-FU and radiation alone did not result in any change in the tumor growth rate or survival of animals compared to saline-treated animals. However, the combination of 5-FU and radiation or 5-FC and radiation resulted in a synergistic decrease in tumor growth rate compared to the single-modality treatments. Despite a dramatic decrease in tumor growth rate, treatment with 5-FU and radiation did not result in a prolonged growth inhibition, for once irradiation was concluded, the tumor promptly resumed growth at a rapid rate. As a result, the overall survival of the group treated with 5-FU and radiation was not enhanced compared to saline-treated animals. This is consistent with clinical studies in which 5-FU and radiation resulted in improved response rates but only infrequently to an increase in overall survival (6). However, the combination of 5-FC and radiation not only exhibited the largest decrease in tumor growth, as measured by MRI, but it also resulted in the greatest enhancement in survival. Finally, the utility of combined 5-FC and radiation was also studied for tumors comprised of different percentages of CD-expressing cells. Inhibition of tumor growth and survival of the animals after treatment with 5-FC and radiation were both enhanced in a manner that was directly proportional to the percentage of CD-expressing cells, but this enhancement was statistically significant only for tumors comprised of at least 25% CD-expressing cells.

In conclusion, these studies suggest a benefit to treatment with the combination of CD/5-FC and radiation in the treatment of head and neck cancer even when only 25% of the tumor was CD-expressing. These results are in accordance with previous studies of this treatment in hind-limb or flank tumors (10, 11), but the response that we saw was less than in those studies. One possible explanation for this decreased response is that the dose of radiation that we were able to administer to the submental region of the mice using this orthotopic tumor model was dictated by the dose-limiting toxicity determined in nontumor-bearing mice, and this dose of radiation was smaller than that administered in the previous studies using CD/5-FC in combination with radiation (10, 11). Thus the limitations to treatment imposed by the normal tissue toxicities in the head and neck region may have inhibited the full efficacy of 5-FC and radiation, although the response was still superior to what we observed for 5-FU and radiation.

Another potential disadvantage of the system described here is its reliance on CD derived from *E. coli*. Recently, we have reported studies both *in vitro* and *in vivo* using the CD gene derived from the yeast *S. cerevisiae* in comparison to the *E. coli* CD (16, 21). Biochemical evaluation of the two enzymes demonstrated that the bacterial protein had a very poor ability to use 5-FC as a substrate when compared to its native substrate, cytosine. The yeast CD, however, used 5-FC with equal efficacy when compared to cytosine, and it was therefore able to convert 5-FC to 5-FU more than 200-fold more efficiently than the bacterial enzyme. *In vivo* comparison of these two enzymes in models of

squamous cell carcinoma of the head and neck (21) or colorectal (16) tumors confirmed the *in vitro* results. Treatment of animals bearing bacterial CD-expressing tumors with 5-FC resulted in growth inhibition but no complete cures. In contrast, there was a greater growth inhibition after 5-FC treatment of yeast CD-expressing tumors and an approximately 60% cure rate. Therefore, the increased ability of yeast CD to use 5-FC as a substrate may translate into a further enhancement of the radiosensitizing effect seen with the combination of the CD/5-FC system and radiation in the treatment of squamous cell carcinoma of the head and neck.

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Enzyme/Prodrug Therapy for Head and Neck Cancer Using a Catalytically Superior Cytosine Deaminase

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ABSTRACT

The use of cytosine deaminase (CD) in conjunction with 5-fluorocytosine (5-FC) has been studied for cancer gene therapy as a means of achieving tumor-specific generation of the toxic metabolite 5-fluorouracil (5-FU). Since 5-FC is frequently used as an antifungal agent, and because it has little or no efficacy as an antibacterial agent, we hypothesized that yeast CD (YCD) might be more efficient at utilizing 5-FC as a substrate and hence be a better choice for a CD/5-FC gene therapy strategy than the typically utilized bacterial CD (BCD). To that end *Saccharomyces cerevisiae* CD was cloned from yeast genomic DNA and expressed *in vitro*. Functional analysis of BCD and YCD expressed in COS-1 cells indicated that BCD and YCD both utilized cytosine with equal efficacy; however, 5-FC was an extremely poor substrate for BCD, with an apparent catalytic efficiency 280-fold lower than that observed for YCD. Retroviral infection of tumor cell lines *in vitro* indicated that the IC_{50} of 5-FC was 30-fold lower in YCD-infected cultures as compared with cultures infected with BCD retrovirus. In addition, when SCCVII murine squamous cell carcinoma cells were infected *in vitro* at low rates of infection ($\leq 10\%$) there was no significant cytotoxicity toward BCD-expressing cells while there was potent cytotoxicity to both YCD-expressing cells and "bystander cells" even at this low level of expression. Finally, stable BCD- or YCD-expressing SCCVII clones were developed and used in an orthotopic immune-competent model of head and neck cancer. Subsequent treatment with 5-FC followed by monitoring of tumor growth by noninvasive magnetic resonance imaging (MRI) and survival of animals indicated a growth delay during the course of 5-FC treatment for BCD-expressing tumors, which quickly regrew at the end of treatment. In contrast, YCD-expressing tumors exhibited not only a growth delay, which was of longer duration, but also in some cases frank tumor regression and complete cures occurred.

OVERVIEW SUMMARY

To improve on the CD/5-FC strategy for enzyme/prodrug therapy for squamous cell cancer of the head and neck the gene for yeast cytosine deaminase (YCD) was cloned. *In vitro* assays indicated that this enzyme utilized 5-FC more efficiently than did the typically used bacterial CD (BCD). Comparison of BCD and YCD expression *in vitro* by retroviral infection revealed that this improved efficiency for conversion of 5-FC to 5-FU translated into more potent cytotoxicity both at lower doses of 5-FC and at lower percentages of expressing cells. Finally, evaluation of this strategy in an *in vivo* model of squamous cell cancer of the head and neck demonstrated that YCD gave an improved therapeutic response with larger and longer lasting growth delays in all cases, and complete cures in others.

INTRODUCTION

THE COMPOUND 5-fluorouracil (5-FU) is commonly used in the treatment of a wide variety of solid malignancies, and it is considered a first-line therapy both alone and in combination therapy in the treatment of squamous cell cancer of the head and neck (SCCHN) (Glick *et al.*, 1980). However, dosing of 5-FU is limited by normal tissue toxicities, primarily to the rapidly dividing tissue in the gastrointestinal tract and bone marrow (Chabner *et al.*, 1996). Therefore, many reports have been

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published on the possibility of using fluorocytosine (5-FC), a nontoxic prodrug of 5-FU, in an enzyme/prodrug strategy for cancer gene therapy (Niculescu-Duvaz et al., 1998). 5-FC is relatively nontoxic when administered to patients; however, it can be readily deaminated by the nonmammalian enzyme cytosine deaminase (CD), resulting in production of 5-FU (Bennett, 1996). Specific transduction of tumor cells with the CD gene followed by systemic administration of 5-FU results in intratumoral production of 5-FU, thus sparing normal tissues from high levels of 5-FU.

The *Escherichia coli* CD gene (BCD) has been studied quite widely *in vitro*, *in vivo*, and in clinical trials focusing on tumor types as varied as colorectal carcinoma, sarcoma, cholangiocarcinoma, and glioma (Niculescu-Duvaz et al., 1998). However, when administering 5-FC as an antifungal agent one of the dose-limiting toxicities is conversion of 5-FC to 5-FU by bacteria resident within the intestinal tract (Diasio et al., 1978). Since these bacteria contain the same CD as that used in previous CD-based enzyme/prodrug strategies it is unlikely that one would ever be able to achieve serum concentrations of 5-FC that are high enough for maximal conversion of 5-FC to 5-FU within the tumor without also resulting in associated systemic toxicities from 5-FU produced by bacteria in the gut. Indeed, although some of the CD/5-FU reports have shown impressive inhibition of tumor growth when F-5C was administered at the maximum tolerated dose, complete cures were infrequently seen (Huber et al., 1993; Trinh et al., 1995). More typically tumor growth was inhibited during 5-FC treatment but resumed once drug therapy was discontinued (Mullen et al., 1994; Pederson et al., 1997). To explore further this enzyme/prodrug strategy we chose to evaluate the cloned yeast cytosine deaminase gene (YCD) in the CD/5-FC system (Erbs et al., 1997). Since 5-FC is frequently used clinically as an antifungal agent we reasoned that yeast cytosine deaminase might be superior at deaminating 5-FC than its bacterial counterpart, resulting in an improved *in vivo* tumor response in gene-dependent enzyme/prodrug therapy (GDEPT).

MATERIALS AND METHODS

Expression plasmids

The expression plasmid for bacterial cytosine deaminase has been previously described (Lawrence et al., 1998). To clone yeast cytosine deaminase, oligonucleotide primers were designed to flank the gene based on the sequence published by Erbs et al. (1997), where the 5′ oligo (5′-CGGAATTCGTC-GACGCAATCATGGTGACAGGGGGAATG) included a consensus Kozack sequence for efficient translation of mRNA and both the 5′ and 3′ oligos (3′-GCGAATTCGTCGACT-TACTACTCACCAATATCTTCAAACC) included flanking *Eco*RI and *Sal*I restriction sites (underlined). YCD was amplified from yeast genomic DNA (kindly provided by A. Welihinda, University of Michigan, Ann Arbor, MI) using Expand DNA polymerase (Boehringer GmbH, Mannheim, Germany). Both BCD and YCD were cloned into the *Eco*RI site of the simian virus 40 (SV40)-based mammalian expression vector pZ (kindly provided the The Genetics Institute, Cambridge, MA). All DNA fragments were sequenced at the University of Michi-

gan DNA Sequencing Core to confirm the lack of any mutations induced by polymerase chain reaction (PCR).

Cell culture and protein expression

SCCVII, COS-1, and ΦnXa cells were cultured under standard conditions in Dulbecc's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine. The pZ.BCD and pZ.YCD expression plasmids were transiently transfected in COS-1 cells by DEAE-dextran transfection and the expressed proteins were metabolically labeled with [^{35}S]Met/Cys (Pro-mix; Amersham, Arlington Heights, IL) according to the protocols previously described (Rehemtulla and Kaufman, 1992). Western blotting was performed as previously described (Hamstra and Rehemtulla, 1999) using anti-CD antibodies (Kievit et al., 1999): rabbit anti-BCD (1:50 × 10^3 dilution) or rabbit anti-YCD (1:1 × 10^6 dilution) was administered, followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (Fisher, Pittsburgh, PA) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Enzymatic assays

CD activity was quantified by percentage conversion of ^{3}H-labeled cytosine or 5-FC according to protocols described elsewhere (Richards et al., 1995). Briefly, transfected COS-1 cells were harvested in assay buffer (100 mM Tris [pH 7.8], 1 mM EDTA) and freeze–thawed three times. To normalize the level of BCD and YCD expressed in each sample parallel plates were transfected, and at the time of harvest these plates were labeled with [^{35}S]Met/Cys. Total cell extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and evaluated by autoradiography. Once developed the photographic film was scanned and the bands quantified using NIH Image software, and the level of expression of BCD and YCD in each sample was then normalized, taking into account the number of methionine and cystine residues present in each enzyme (13 for BCD and 12 for YCD). These normalized expression levels were then used to adjust the amount of protein extracts used for activity assays. Over the course of three experiments the level of BCD and YCD in each sample varied by ±15%, and this factor was taken into account in all calculations.

For cytosine and 5-FC conversion assays 100 mM cytosine or 5-FC was spiked with 0.5 mM [6-^{3}H]cytosine or 5-[6-^{3}H]FC (1 μCi/mmol; Moravek Biochemicals, Brea, CA) and then diluted with buffer yielding final concentrations of cold cytosine or 5-FC from 0.2 to 40 mM. Each assays was performed in a 30-μl reaction volume to which 0.5 to 30 μg of protein extract was added, and the reactions were then incubated for 15 min to 2 hr at 37°C, at which time they were quenched by the addition of 1 M acetic acid. The [^{3}H]uracil or 5-[^{3}H]FU produced was isolated by elution from an SCX Bond Elute column (Varian, Harbor City, CA) and counted on a liquid scintillation counter. An equal amount of reaction mixture at each dilution was also counted in order to determine total counts (TCs). Percentage conversion was calculated as (cpm of ^{3}H-labeled uracil or 5-FU/cpm of TC) × 100. The maximum conversion that could be measured with this assay was 75–80%. Percentage conversion values were then used to calculate the actual pro-

duction of uracil or 5-FU per microgram of protein extract per minute of reaction time. These data were plotted with Graph-Pad Prism software (GraphPad Software, San Jose, CA) and apparent K_m and apparent V_{max} values calculated based on non-linear regression analysis. All assays were performed in quadruplicate with samples derived from two separate transfections.

Retroviral production and infection

To produce CD retroviruses the BCD or YCD cDNAs were excised from the respective expression plasmids by *Eco*RI digest and cloned into the *Eco*RI site of the Lzr.Neo expression plasmid (Hamstra *et al.*, 1999a). This retroviral construct, derived from Lzrs.pBMN.LacZ (G. Nolan, Stanford, CA), drives expression of the transgene from the Moloney leukemia virus long terminal repeat (LTR). In addition, downstream of the transgene is an internal ribosomal entry site (IRES) followed by the neomycin phosphotransferase gene (*neo^R*), thus allowing for selection of infected cells on the basis of resistance to G418. The vector also has an independent puromycin expression cassette for selection of retroviral producer lines. The ΦnX-ampho retroviral packaging cell line (courtesy of G. Nolan, Stanford, CA) was transfected with the representative plasmids by calcium phosphate precipitation, and 48 hr after transfection producer cells were selected in puromycin (0.5 μg/ml; Sigma, St. Louis, MO). Retroviral supernatants were generated by plating puromycin-selected producer cells at a density of 40,000 cells/cm^2 in 100-mm plates and culturing at 32°C for 4 days, harvesting the medium daily. Pooled supernatants were filtered through a 0.4-μm pore size filter, aliquotted, and frozen at -70°C. Cells were infected with these retroviral supernatants supplemented with Polybrene (16 μg/ml; Sigma). LacZ-, BCD-, and YCD-expressing viral batches were titered on SCCVII cells on the basis of G418 resistance (GIBCO-BRL, Gaithersburg, MD) for the two CD viruses and on the basis of β-galactosidase staining for the LacZ virus, yielding titers of $2.4 \times 10^6 \pm 0.7 \times 10^6$, $4.0 \times 10^6 \pm 1.2 \times 10^6$, and $5.2 \times 10^6 \pm 1.4 \times 10^6$ CFU/ml for LacZ-; BCD-; and YCD-expressing viruses, respectively.

Growth inhibition and cytotoxicity assays

For some experiments growth inhibition was evaluated by the sulforhodamine B assay (SRB) (Skehan *et al.*, 1990). SCCVII cells in 96-well plates were infected with LacZ, BCD, or YCD retroviruses, and cellular growth after 5-FC treatment was evaluated on the basis of total cellular protein staining with SRB as measured by a 96-well microtiter plate reader. Briefly, 12 to 18 hr prior to infection SCCVII cells were seeded at a density of 3000 cells/cm^2 in 96-well plates, and 24 hr after infection the medium (200 μl) was changed to that supplemented with vehicle (phosphate-buffered saline, PBS) or 5-FC (Sigma). Cells were treated with 5-FC for 72 hr, in order to allow untreated cells to reach confluence, and at that point the cells were fixed and stained according to Skehan *et al.* (1990) and the plates read on a microtiter plate reader using a 490-nm filter (Vmax; Molecular Devices, Sunnyvale, CA). Data plotted represent the mean and standard deviation of eight replicate wells.

For other experiments cellular cytotoxicity was evaluated by a colony formation assay (CFA) as previously described

(Lawrence, 1988). Cells were seeded in 60-mm dishes at a density of 2000 cells/cm^2, and 18–24 hr later they were infected with retroviral supernatants at titers ranging from 3×10^4 to 2×10^6 CFU/ml for 18–24 hr, at which time the medium was changed to that supplemented with vehicle (PBS) or 5-FC. Cells were treated with the 100 μM 5-FC for 48 hr, at which point they were plated for colony formation, and after 7–10 days the dishes were fixed and stained with crystal violet before counting. Data plotted represent the mean and standard error of at least four experiments.

Stable cell lines

BCD- and YCD-expressing SCCVII stable cell lines were established by retroviral infection and limiting dilution in 96-well plates. Ten to 20 clones were assayed for each enzyme and the best expressing clones verified by growth inhibition assay, cytosine and 5-FC conversion, and Western blot. The sensitivity of these cell lines to 5-FC as assessed by SRB assay was similar to that seen for the pooled retrovirally transduced populations.

In vivo model

Animal experiments, including designations for survival outcomes, were approved by the University of Michigan Committee on Use and Care of Animals. A modified form of an immune-component model of SCCHN was used (O'Malley *et al.*, 1997a). Briefly, SCCVII cells expressing BCD or YCD growing in culture were trypsinized, washed in PBS, counted, and resuspended at a concentration of 4×10^8 cells/ml in PBS. Fifty microliters of this cell solution, containing 2×10^6 cells, were then injected into the submental compartment of C3H mice (6–8 weeks; Charles River Laboratories, Wilmington, MA) along the midline, using an external approach (Dinesmen *et al.*, 1990). The presence of tumors was verified by magnetic resonance imaging (MRI) 3–4 days after intraperitoneal injection and treatment with 5-FC (500 mg/kg daily) was initiated on day 5 after tumor cell implantation and continued for a total of 10 days. Mice were weighed every 2–3 days and were euthanized if they lost >30% of their body weight. The significance for survival between treatment groups was evaluated by Student's *t* test for unpaired data.

Growth of tumors during and after treatment with 5-FC was monitored by sequential MRI studies over 3 weeks, using a Varian MRI system (Varian Instruments, Fremont, CA) equipped with a 7-T, 18.3-cm horizontal bore magnet (Oxford Instruments, Oxford, UK) with actively shielded gradients. In brief, for MRI examination, mice were anesthetized with sodium pentobarbital (60–70 mg/kg, intraperitoneal) and positioned within a small radio frequency quadrature coil (USA Instruments, Aurora, OH). A single-slice gradient-recalled echo image was acquired with 1-mm "saturation crosshairs" imprinted on the axial and transverse images to facilitate rapid and reproducible positioning on the animal. Multislice transverse T2-weighted images were acquired by using a spin echo sequence with the following parameters: 3.5-sec repetition time, 60-msec echo time, field of view = 3×3 cm using a 128×256 matrix, slice thickness = 1.0 mm, slice separation = 0 mm, number of slices = 24, and four signal averages per phase encode step.

In vivo SCCVII tumor volumes were quantitated from the

multislice MR images by electronically outlining the region of interest with image processing software (Advanced Visual Systems, Waltham, MA) as previously described (Ross et al., 1998). The number of pixels was converted to a volume and the total volume of the tumor was calculated by summing the volumes from individual slices. Tumor volumes obtained over time were used to calculate tumor growth rates for individual animals.

RESULTS

Cloning and expression of YCD and BCD

To compare YCD with BCD for use as an enzyme/prodrug strategy for cancer gene therapy the YCD gene was amplified from *Saccharomyces cerevisiae* genomic DNA, using PCR primers designed on the basis of the published sequence (Erbs et al., 1997). The PCR product obtained was cloned into the expression vector pZ, and four distinct clones were isolated; one of them (clone 14) was sequenced to confirm the identity of the cDNA and to rule out any PCR artifacts. The cDNA was identical in all respects to that reported by Erbs et al. containing a 459-bp open reading frame encoding a protein of 158 amino acids. Comparison of YCD and BCD revealed that there is no apparent homology (<10%) between S. cerevisiae CD and E. coli CD at either the nucleotide or protein level.

Equal amounts of BCD and YCD expression plasmids (8 μg) were transfected into COS-1 cells and 48 hr posttransfection the cells were labeled with [^{35}S]]Met/Cys, resolved by SDS-PAGE, and evaluated by autoradiography (Fig. 1A). The neomycin phosphotransferase (*neo^r*) gene product is evident as a band with an apparent molecular mass of 22 kDa (marked with an asterisk in Fig. 1A), which is present in both of the transfected cell extracts but not in that from mock-transfected

cells (Fig. 1A, lanes 1–3). The level of expression of the neomycin phosphotransferase protein, which was expressed downstream of both CD genes from a bicistronic mRNA, thus functions as an internal control for rate of transfection, mRNA synthesis, and expression. By SDS-PAGE BCD appears as a protein with a molecular mass of approximately 48 kDa (Fig. 1A, lane 2, marked with a solid arrow), which is consistent with previous reports (Katsuragi et al., 1986). In contrast, YCD migrates as a protein with a molecular mass of only 17.5 kDa (Fig. 1A, lane 3, marked with a dashed arrow), which is also consistent with previous reports (Katsuragi et al., 1989). Western blots with BCD-specific (Fig. 1B) and YCD-specific (Fig. 1C) antisera were performed and revealed that the proteins expressed are indeed BCD and YCD, respectively. The lack of cross-reactivity between the two polyclonal antibodies is further confirmation of the lack of homology between the two proteins.

YCD utilizes 5-FC more efficiently than BCD

To characterize both proteins functionally, the BCD and YCD expression plasmids were transfected into COS-1 cells. Forty-eight hours after transfection cell pellets were harvested and lysates made, the amount of CD in each sample was normalized using a parallel transfection, as described in Materials and Methods, and this normalized amount of cell extract was used in each assay. When 0.5 to 8 μg of cell extract from BCD- or YCD-transfected cells was incubated with 1 mM cytosine for 2 hr a dose-dependent conversion of [^{3}H]cytosine to [^{3}H]uracil was observed. Under these conditions both enzymes reached saturation (80–85% conversion) at between 2 and 4 μg of cell extract (Fig. 2). In contrast, when BCD and YCD cell extracts were incubated for 2 hr with 1 mM 5-FC there was a marked difference between BCD and YCD extracts in the production of 5-[^{3}H]FC. YCD extracts converted 5-FC to an ex-
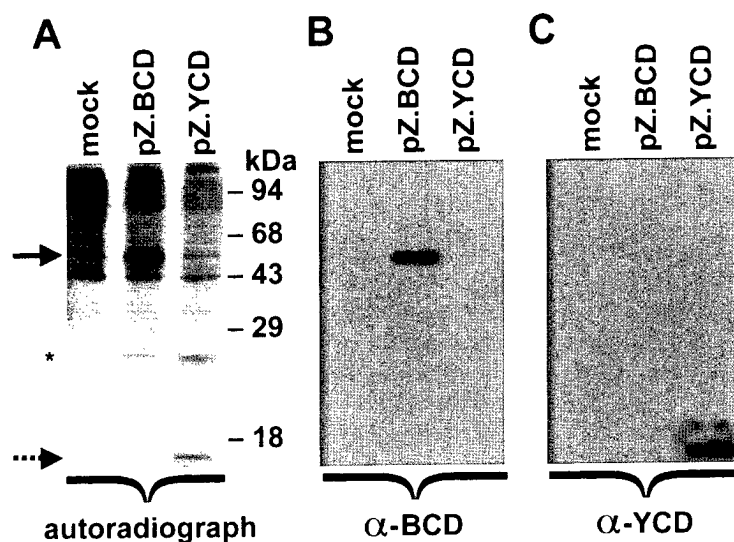

FIG. 1. Expression of BCD and YCD in COS-1 cells. COS-1 cells were transiently transfected with BCD or YCD expression plasmids or in the absence of DNA (mock). Transfected cells were analyzed by [^{35}S]Met-Cys labeling, SDS-PAGE, and autoradiography (**A**). Bands representing BCD or YCD are indicated by solid and dashed arrows, respectively; and the *neo^r* gene product is marked with an asterisk (*). Western blots were also performed with anti-BCD or anti-YCD antisera (**B** and **C**).
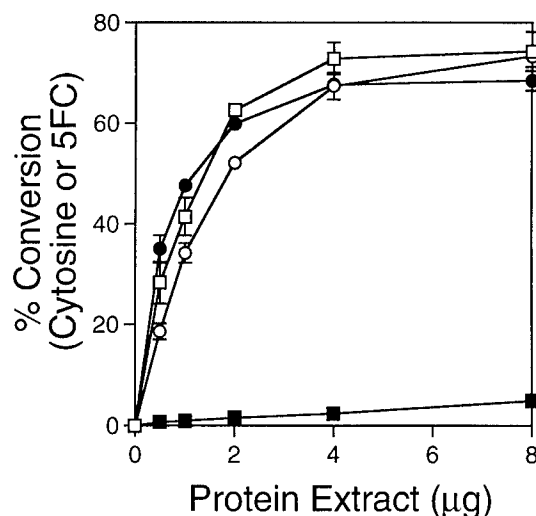
FIG. 2. YCD converts 5-FC to 5-FU more efficiently than BCD. Cell extracts from transiently transfected COS-1 cells were used to measure the conversion of 1 mM cytosine or 5-FC in 2 hr, as described in Materials and Methods. Conversion of cytosine by BCD (open squares) or YCD (open circles), and conversion of 5-FC by BCD (closed squares) and YCD (closed circles) are shown. Data represent the means and standard errors of four experiments.

tent similar to the conversion observed for cytosine, with saturation again achieved at between 2 and 4 μg of cell extract (Fig. 2). In contrast, BCD had a poor capacity to utilize 5-FC, and even when 8 μg of extract from BCD-transfected cells was used the conversion was still less than 5% and saturation was not achieved (Fig. 2). The poor utilization of 5-FC by BCD is consistent with previous reports (Katsuragi *et al.*, 1986).

To better characterize the difference between the utilization of 5-FC by BCD and YCD, more detailed kinetic experiments were performed. The concentration of cytosine or 5-FC was varied from 0.2 to 40 mM and the amount of produced uracil or 5-FU was quantified. Data were plotted as the concentration of cytosine or 5-FC versus the amount of uracil or FU produced per microgram of protein extract per minute, and the apparent K_m and apparent V_{max} were calculated on the basis of nonlinear regression analysis (Table 1). The apparent K_m values of BCD and YCD for cytosine were within twofold of each other. 5-FC was an extremely poor substrate for BCD, with a K_m >40-fold higher than YCD. These values are consistent with the con-

version efficiencies observed (Fig. 2). When the "catalytic efficiencies" of the two enzymes, defined as the V_{max}/K_m ratio, were calculated it became apparent that the two enzymes had similar efficiencies for cytosine (V_{max}/K_m of 2.3 and 3.0 for BCD and YCD, respectively). In contrast, YCD utilized 5-FC as a substrate 280-fold more efficiently than BCD (V_{max}/K_m of 0.045 and 12.9 for BCD and YCD, respectively).

YCD sensitizes cells to 5-FC at lower doses than BCD

Since YCD was found to be functionally superior to BCD in converting 5-FC to 5-FU, we next evaluated whether these enzymatic differences had an impact on tumor cell growth *in vitro* and *in vivo*. Matched retroviral vectors encoding BCD and YCD were developed and used at equal titers (2 $\times$ 10^6 CFU/ml) to infect SCCVII cells. At this titer the infection rate as determined by number of G418-resistant colonies was 42 $\pm$ 4% in SCCVII cells for BCD virus and 38 $\pm$ 5% in SCCVII cells for YCD virus (data not shown, and see Fig. 4). Western blots of SCCVII cell extracts from LacZ-, BCD-, or YCD-expressing retrovirus-infected cells demonstrated that retroviral transduction does indeed result in the production of the expected proteins (Fig. 3A).

An SRB assay on cells grown in 96-well plates was used to evaluate growth inhibition of SCCVII cells after infection with LacZ-, BCD-, or YCD-expressing retroviruses and treatment with 5-FC for 72 hr (Fig. 3B). When SCCVII cells were infected with the control LacZ retrovirus and treated with up to 10 mM 5-FC cellular growth was not significantly inhibited, and the concentration of 5-FC necessary to inhibit cellular growth by 50% (IC$_{50}$) could not be determined (data not shown). In contrast, the IC$_{50}$ after BCD-expressing retroviral infection and 5-FC treatment was 0.6 mM in SCCVII cells. More importantly, infection of SCCVII cells with YCD retrovirus followed by 5-FC treatment resulted in a 30-fold shift in IC$_{50}$ compared with BCD infection to 0.02 mM.

At low levels of infection YCD exhibits a greater "bystander effect" than BCD

Since most common gene delivery vectors are currently only able to transduce approximately 10% of the cells within the tumor after *in vivo* infection, we next examined the efficacy of BCD and YCD *in vitro* under conditions where the percentage of cells infected was varied. For this experiment a colony formation assay was utilized to assess the clonigenic growth potential of cells after retroviral infection and 5-FC treatment. This

TABLE 1. APPARENT KINETIC CONSTANTS FOR BCD AND YCD WITH CYTOSINE AND 5-FC[a]

	Cytosine			5-FC		
	$K_m(app)$ ($1/m$M)	$V_{max}(app)$ (μmol/min /mg lysate)	$V_{max}(app)/K_m(app)$ ($1/\mu$g lysate/min)10^{-3}	$K_m(app)$ ($1/m$M)	$V_{max}(app)$ (μmol/min /mg lysate)	$V_{max}(app)/K_m(app)$ ($1/\mu$g lysate/min)10^{-3}
BCD	2.3 $\pm$ 0.4	5.4 $\pm$ 0.2	2.3	31 $\pm$ 7.4	1.4 $\pm$ 0.2	0.045
YCD	4.5 $\pm$ 0.7	13.5 $\pm$ 0.6	3.0	0.7 $\pm$ 0.14	9.0 $\pm$ 0.4	12.9

[a]Hydrolysis of cytosine or 5-FC by cell extracts from transiently transfected COS-1 cells expressing BCD or YCD. Cell lysates were normalized for gene expression as described in Materials and Methods and the conversion of [^{3}H]uracil or 5-[^{3}H]FU was quantified. Data represent the means $\pm$ standard error of four experiments.
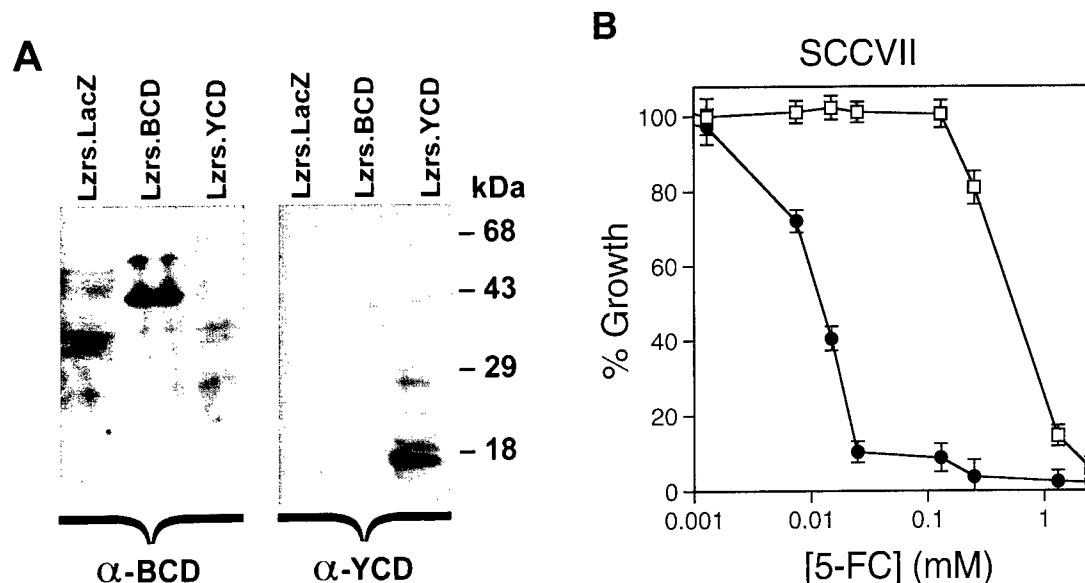
FIG. 3. YCD sensitizes cells to 5-FC more efficiently than BCD after retroviral infection. SCCVII cells, a murine squamous cell carcinoma line, were infected with LacZ-, BCD-, or YCD-expressing retroviruses (2×10^6 CFU/ml). Forty-eight hours postinfection cell lysates were made, resolved by SDS-PAGE, and Western blots performed using anti-BCD or anti-YCD antiserum (**A**). At the same time SCCVII cells were seeded in 96-well plates and infected with BCD-expressing (open squares) or YCD-expressing (closed circles) retroviruses (2×10^6 CFU/ml) before treatment with 5-FC for 72 hr and evaluation by SRB growth inhibition assay. Data represent the means ± the standard deviations of at least eight replicate wells (**B**).

assay more accurately simulates *in vivo* cytotoxicity, while offering greater sensitivity than the SRB assay, and also allows for direct measurement of the percentage of infected cells in parallel to the cytotoxicity assays. All treatments utilized a 48-h treatment with 100 μM 5-FC, because preliminary experiments indicated maximal toxicity to BCD-expressing SCCVII cells at this dose and time with no increase in cytotoxicity with either increased duration of drug exposure or increased concentration of 5-FC (data not shown).

By decreasing the titers of the BCD and YCD retroviruses the infection rates were decreased from 40% to less than 5% for both BCD and YCD. At higher rates of infectivity, approaching 40%, where the level and activity of the enzyme would not be expected to be rate limiting, there was potent cytotoxicity to cultures treated with 100 μM 5-FC for 48 hr, with a surviving fraction of between 0.1 and 0.01, in both BCD- and YCD-expressing cultures (Fig. 4). However, when rates of expression dropped below 20% of the total cellular population, where enzyme is most likely rate limiting, the cytotoxicity in BCD-expressing cultures rapidly decreased to such a point that little cytotoxicity was seen below 10% expression (surviving fraction [SF]>0.75). In contrast, the cytotoxicity to YCD-expressing cultures, using the same dose of drug, was significantly higher, with a surviving fraction of <0.1 even with expression by less than 5% of the cells in culture. Thus, the improved catalytic efficiency witnessed for YCD, when compared with BCD in biochemical assays, appears to sensitize infected cells to lower concentrations of 5-FC and at lower transduction efficiencies, which are consistent with current gene therapy vectors *in vivo*.
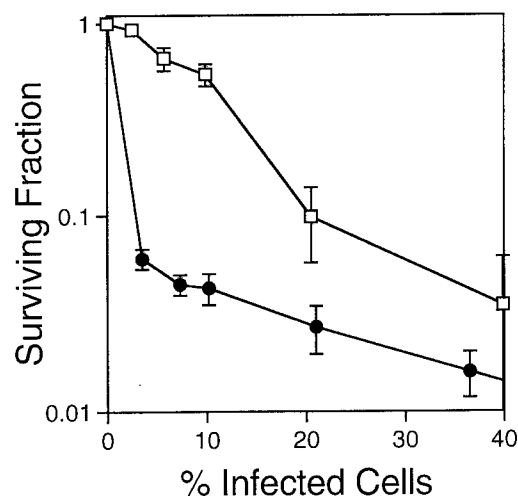

FIG. 4. YCD-expressing cells exhibit a more potent "bystander effect" than BCD-expressing cells. SCCVII cells were infected with increasing titers of BCD-expressing retrovirus (open squares) or YCD-expressing retrovirus (closed circles) and then treated with 5-FC (100 μM) for 48 hr before plating for colony formation assay. Data are plotted as surviving fraction as a function of percent infected cells and represent the means ± the standard errors of four experiments.

YCD results in enhanced growth inhibition and tumor regression in vivo

Since YCD appeared to be a better enzyme at deaminating 5-FC as evaluated by functional assays and by *in vivo* growth inhibition and cytotoxicity assays, we next wanted to determine if this also held true in an *in vivo* model of SCCHN. Stable SC-CVII cell lines expressing BCD and YCD were established, and *in vitro* SRB growth inhibition assays were performed to verify that the growth characteristics of the BCD- and YCD-expressing lines in the absence and presence of 5-FC were similar to that of the retrovirally infected cultures (Fig. 5). Tumors were established in C3H mice by injection of 2×10^6 BCD- or YCD-expressing SCCVII cells into the submental space. Since SCCVII cells are syngeneic for C3H mice this model allows for the evaluation of this enzyme/prodrug strategy in an immune-competent animal model of head and neck cancer (O'Malley et al., 1997a).

5-FC treatment was initiated 5 days after implantation, when the presence of tumors had been established by MRI. Tumors were of similar size between groups, with an average tumor volume of 11.5 ± 1.4 mm^3. After the initial MRI one animal was randomly selected from each group to serve as a marker animal and was submitted for sequential MRIs over the 25 days after implantation. Representative MRI slices from each of the marker animals are shown (Fig. 6A–D). These MRI studies demonstrate that this orthotopic tumor grows with a nonspherical and endophytic phenotype, which renders standard caliper-based measurements nonviable. However, the MRI studies were used to measure the size of the tumors both during and after treatment with 5-FC (500 mg/kg, once daily for 10 days); data were plotted as relative tumor volume as a function of days postimplantation (Fig. 6E).
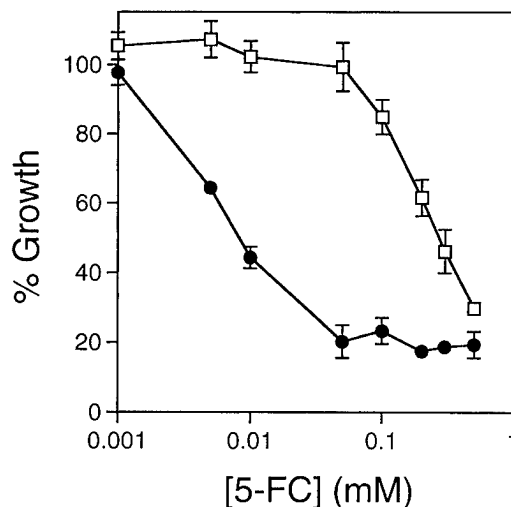
Untreated BCD and YCD tumors grew rapidly, with a doubling time of about 1.0 and 2.1 days, respectively. In contrast, the BCD-expressing tumor that was 5-FC treated (days 5 through 14 postimplantation) had a significant reduction in tumor growth, with a doubling time that was 2.4 times longer than that of the untreated BCD-expressing tumor. However, this growth inhibition was short-lived since the tumor resumed an active growth rate once 5-FC treatment was discontinued. In contrast, the YCD-expressing tumor experienced a greater growth inhibition both during and after 5-FC treatment, with a doubling time that was 5.2 times longer than that of the untreated tumor. In fact, during the early part of 5-FC treatment the YCD-expressing tumor regressed to such a point that it was no longer detectable by MRI.

At the end of the MRI studies (25 days after tumor implantation) the untreated mice bearing BCD- or YCD-expressing tumors had died or were euthanized owing to excessive weight loss. At the time of death (22 and 25 days postimplantation for the BCD and YCD tumor-bearing animals, respectively) the untreated tumors were 20 and 31 times larger than the initial measurement at the start of treatment (249 versus 12.5 mm^3 and 234 versus 7.5 mm^3 for untreated BCD and YCD tumors, respectively) (Fig. 6A, C, and E). As discussed above, the tumor from the animal that was implanted with BCD-expressing SC-CVII cells experienced a growth delay during 5-FC treatment; however, by the end of the MRI studies (day 25 postimplantation) the tumor was 15 times larger than at the start of treatment (195.8 versus 12.6 mm^3) (Fig. 6B and E), and it was necessary to euthanize the animal. Finally, the YCD-expressing tumor treated with 5-FC had a much slower growth rate both during and after 5-FC treatment, and at the end of the MRI studies (25 days postimplantation) this tumor was still only 4.4 times larger than at the start of therapy (57.8 versus 13.3 mm^3) (Fig. 6D and E). With a tumor volume of 57.8 mm^3 the YCD-expressing tumor that was 5-FC treated was considerably smaller than tumors from any of the other mice. It was on average 4.1 times smaller than the untreated tumors and 3.3 times smaller than the BCD-expressing tumor that was 5-FC treated.

MRI studies offer a noninvasive, quantitative, and dynamic method for evaluating tumor size, growth, and response to treatment (Ross et al., 1998). However, these studies are limited by the number of animals that can be evaluated at once and the time and expense of the studies. Therefore, as a second criterion to evaluate this *in vivo* model we measured the increase in survival after 5-FC treatment of animals bearing BCD- and YCD-expressing tumors (Fig. 7). There was no significant difference between the survival of animals implanted with BCD- or YCD-expressing tumors that were untreated ($p > 0.08$). Untreated BCD and YCD tumor-bearing mice had a time to 50% survival of 20 and 27 days, respectively, and both populations had no surviving animals past day 38. 5-FC treatment of animals bearing BCD-expressing tumors resulted in a significant increase in survival ($p < 0.02$), and the time to 50% survival doubled from 20 days for untreated animals bearing BCD-expressing tumors to 40 days after 5-FC treatment. However, there were still no surviving animals past 82 days. Finally, animals implanted with YCD-expressing tumors that received 5-FC treatment had a 60% survival rate (three of five) at 120 days postimplantation. This increase in survival for the animals bearing YCD-expressing tumors treated with 5-FC was significant

FIG. 5. A stable cell line expressing YCD is more sensitive to 5-FC than a cell line expressing BCD. Stable BCD-expressing (open squares) or YCD-expressing (closed circles) SCCVII cell lines were developed and tested for sensitivity to 5-FC by SRB assay as described in Materials and Methods. Data represent the means and standard deviations of at least eight replicate wells.
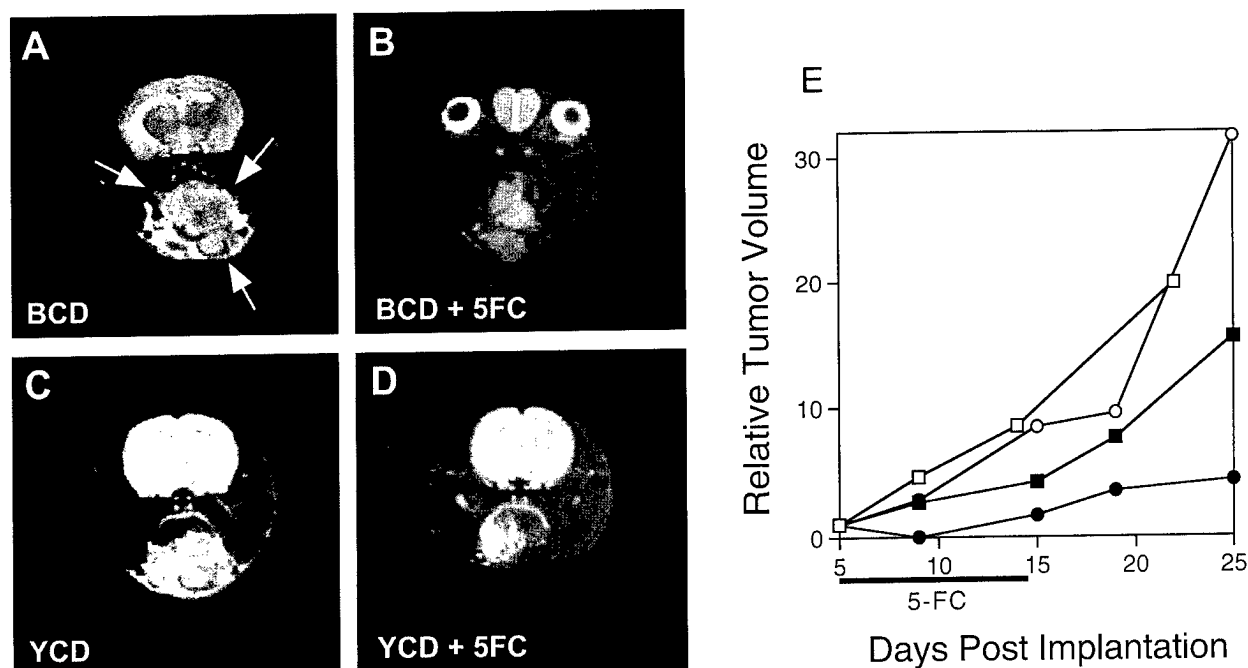
FIG. 6. YCD-expressing tumors *in vivo* exhibit an enhanced growth inhibition after treatment with 5-FC. Orthotopic tumors were developed by injecting 2×10^6 BCD- or YCD-expressing SCCVII cells into the submental space of C3H mice (as described in Materials and Methods). Tumor growth was subsequently evaluated by MRI of representative animals starting 5 days after implantation and continuing for 20 more days. T2-weighted MR images through the largest cross-section of the tumors are displayed for BCD-expressing tumors untreated (**A**) and 5-FC treated (**B**) and for YCD-expressing tumors untreated (**C**) and 5-FC treated (**D**). All images were taken from the last day of MRI studies (day 25 after tumor cell implantation) except for the untreated BCD tumor (**A**), which was from day 22 (the last MRI before the mouse expired). For reference purposes the dimensions of the tumor in (**A**) are indicated with arrows. A graph of relative tumor volume as a function of days postimplantation is plotted on the basis of volumetric data acquired from MRI images (**E**). The course of 5-FC treatment (500 mg/kg, once daily for 10 days) is indicated with a bar. BCD tumors untreated (open squares) or 5-FC treated (closed squares) and YCD tumors untreated (open circles) or 5-FC treated (closed circles).

when compared with both untreated mice bearing YCD-expressing tumors ($p < 0.009$) and when compared with mice bearing BCD-expressing tumors that were 5-FC treated ($p < 0.025$).

DISCUSSION

There are a variety of approaches under development to circumvent the problem of nonselective toxicity of chemotherapeutic agents. One of these approaches depends on prodrugs, chemicals that are pharmacodynamically and toxicologically inert even at high doses, but that can be converted *in vivo* to highly active species at tumor sites owing to the presence of a specific enzymatic activity. A number of gene-based approaches have been developed as enzyme/prodrug therapies, and these have been labeled *gene-dependent enzyme prodrug therapy* (GDEPT) (Niculescu-Duvaz *et al.*, 1998).

A prototype of GDEPT is the herpes simplex virus thymidine kinase (HSV TK) in conjunction with the antiviral drug ganciclovir (GCV), which is preferentially phosphorylated by HSV TK and subsequently by cellular kinases to GCV triphosphate, an inhibitor of DNA synthesis (Moolten, 1986). This system has undergone widespread testing (Moolten, 1986; Moolten

and Wells, 1990; Culver *et al.*, 1992). However, one potential problem with the HSV TK-based system is that the toxic ganciclovir metabolites are not membrane permeable and thus depend on gap-junctional intracellular communication (GJIC) for passage from one cell to the next (Fick *et al.*, 1995; Mesnil *et al.*, 1996; Wygoda *et al.*, 1997). As a result there is little cytotoxicity in this system when less than 50% of the cells are expressing HSV TK (Wygoda *et al.*, 1997). In contrast, the 5-FU generated from the CD/5-FC system is permeable to the cell membrane and readily diffuses between transduced and nontransduced cells independent of the presence or absence of GJIC (Trinh *et al.*, 1995). This freely diffusing and permeable metabolite may account for the high "bystander effect" that we reported here and that has been seen in other studies of the CD/5-FC system both *in vitro* and *in vivo* (Trinh *et al.*, 1995; Lawrence *et al.*, 1998).

The first studies using bacterial CD utilized a model for colorectal cancer (Huber *et al.*, 1993, 1994); tumors regressed after treatment with 5-FC at 500 mg/kg for 10 consecutive days, but a relapse was observed in 70% of the tumors once treatment was discontinued. As a direct comparison to the HSV TK system these investigators later demonstrated that only 4% of the tumor cells had to be transduced with the CD gene in order to observe tumor regressions, whereas there was no clini-

cal response when as much as 10% of the cells were expressing HSV TK. Again, however, a relapse in 40% of the tumors was observed when 5-FC treatment was stopped (Trinh et al., 1995). In contrast, others have reported only a small tumor growth delay in CD-expressing fibrosarcomas (Mullen et al., 1994) and cholangiocarcinoma (Pederson et al., 1997) when mice were treated with 5-FC. These results are comparable to the limited tumor growth delay that we observed during 5-FC treatment of mice bearing BCD-expressing SCCVII tumors.

One potential explanation for the limited responses seen in vivo when using the combination of BCD/5-FC is that the maximal tolerated serum concentration of 5-FC is significantly below the K_m of BCD. We utilized the treatment schedule of 500 mg/kg daily for 10 days on the basis of reports in the literature (Huber et al 1993, 1994) and our own preliminary experiments, which determined that this was the maximal tolerated dose of 5-FC over a 10-day treatment schedule. In addition, many previous studies of BCD/5-FC for GDEPT utilized this treatment protocol and we, therefore, felt that for the sake of comparison it was useful to maintain a schedule similar to that previously reported. In humans it has been determined that conversion of 5-FC to 5-FU, by bacteria in the gut, becomes dose limiting for 5-FC serum concentrations above 700–800 μM (Diasio et al., 1978; Bennett, 1996). This value is approximately 40-fold lower than the apparent K_m of BCD that we determined with BCD produced in COS-1 cells after transient transfection. However, using the same assay YCD had an apparent K_m (700 $\pm$ 140 μM) that lies within the reported clinically achievable serum concentration of 5-FC in humans. The kinetic data reported here are also similar to what we obtained using BCD and YCD pro-

duced in E. coli and purified as fusion proteins (Kievit et al., 1999).

In this article we did not directly measure the serum concentrations of 5-FC or 5-FU after intraperitoneal injection. However, we have completed studies detailing the noninvasive quantitation of cytosine deaminase transgene expression by ^{19}F magnetic resonance spectroscopy of 5-FC, 5-FU, and its metabolites in a colon carcinoma model for GDEPT (Stegman et al., 1999). After a single intraperitoneal injection of 5-FC (1000 mg/kg) into non-tumor-bearing mice, peak serum levels of 7.2 $\pm$ 0.1 mM, as determined by gas chromatography and mass spectroscopy, were achieved 45 min after injection, and the clearance of 5-FC from the serum could be described by a single exponential with a half-life of 44.7 min. To facilitate ^{19}F magnetic resonance spectroscopy in this study it was necessary to use a single dose of 5-FC that was twice as large as we were able to administer safely on a daily schedule. Even taking this increased 5-FC dose into account, the serum levels that we determined analytically suggest that peak serum concentrations of 5-FC can be achieved in mice in the low millimolar range after 5-FC is administered intraperitoneally at 500 mg/kg. This 5-FC serum level would be at or above the apparent K_m of YCD but still significantly below the K_m of BCD. These results, therefore, suggest that YCD, with an apparent K_m for 5-FC within the range obtainable in the serum of both humans and mice, may be far more effective in utilizing 5-FC in vivo than BCD. This superior utilization of 5-FC by YCD most likely explains the better clinical response that we obtained when evaluating this enzyme/prodrug strategy in an orthotopic model of SCCHN.

In addition, we also evaluated the efficacy of 5-FU treatment of mice bearing submental SCCVII tumors at the maximum tolerated dose (25 mg/kg administered intraperitoneally daily for 10 days), and both by MRI and survival there was no therapeutic advantage when compared with untreated animals (data not shown; and Hamstra et al., 1999b). Thus, not only is the combination of YCD/5-FC superior to the comparable BCD/5-FC therapy, but it also offers an improved therapeutic index and response over conventional treatment with 5-FU. As further validation of the superiority of YCD over BCD, we reported similar GDEPT experiments comparing BCD- and YCD-expressing cell lines in a flank tumor model of colorectal cancer in nude mice (Kievit et al., 1999). The results in the flank tumor model confirm what we witnessed here, with an overall survival rate of 60% for 5-FC-treated animals bearing YCD-expressing tumors, but only a growth delay and no long-term survivors for animals implanted with BCD-expressing tumors that received 5-FC.

The orthotopic in vivo model of head and neck cancer utilized in these studies has many advantages over the commonly used flank tumor models. Submental implantation mimicks the normal growth and phenotype of SCCHN seen in humans, including invasion of soft tissue and bone, local–regional metastasis, and the infringement on vital structures within the neck and airway with resulting cachexia (O'Malley et al., 1997a). In addition, the presence of an intact immune system allows for evaluation of potential synergistic effects of the immune response in combination with the gene therapy strategy (O'Malley et al., 1997b). Also, the toxicities of both the disease and the treatment more closely resemble the clinical scenario en-
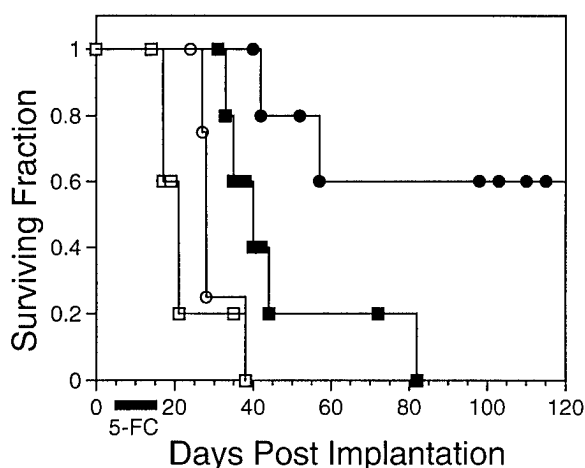

FIG. 7. Animals bearing YCD-expressing tumors have enhanced survival after 5-FC treatment. Submental tumors were developed in mice and 5-FC treatment was performed as described in Materials and Methods and in the caption to Fig. 6. A plot of surviving fraction as a function of days postimplantation for mice bearing BCD-expressing tumors untreated (open squares, $n = 5$), BCD-expressing tumors 5-FC treated (closed squares, $n = 5$), YCD-expressing tumors untreated (open circles, $n = 4$), or YCD-expressing tumors 5-FC treated (closed circles, $n = 5$) is shown. The course of 5-FC treatment (500 mg/kg, once daily for 10 days) is indicated with a bar.

countered with head and neck cancer, and, thus, such an orthotopic model may be vital for future preclinical evaluation of GDEPT strategies for this disease. However, physical measurement and quantification of this tumor model are difficult owing to the nonspherical and locally invasive nature of the tumor (see Fig. 6A–D), which necessitates surgical dissection in order to visualize and quantify the tumor burden. Therefore, we chose to use an MRI-based assay as a first assessment of tumor response and clinical efficacy since MRI tumor volumes can be monitored dynamically throughout the course and after treatment without the need for invasive surgical procedures (Ross *et al.*, 1998). The MRI data, although constrained by the number of animals that we could effectively evaluate, were complementary with and more importantly predictive of the results obtained in standard survival experiments. These results suggest that in the future more complete MRI-based evaluation may provide a rapid and quantitative means to evaluate tumor treatment outcomes in this orthotopic model of SCCHN.

Owing to the limitations of BCD and the inability of most groups to elicit complete cures with the traditional CD/5-FC system, many studies that have been published more recently combine CD/5-FC with other therapeutic protocols such as concurrent radiotherapy (Khil *et al.*, 1996; Hanna *et al.*, 1997; Pederson *et al.*, 1997) or dual enzyme/prodrug therapy combining the CD/5-FC and HSV TK/ganciclovir approaches (Rogulski *et al.*, 1997). In most cases these dual therapies were synergistic and able to elicit a greater response that the CD/5-FC approach alone. It, therefore, seems likely that the improved response that we have shown here with YCD can be applied to these combined modality therapies to further enhance the response. Indeed, preliminary data combining the YCD/5-FC system with concurrent radiotherapy in the model of SCCHN described here support this notion (unpublished data).

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	2001	M.D. The University of Michigan Medical School
	1993:	BA, Biochemistry Calvin College. Grand Rapids, MI

Honors & Awards

1997-2000	Doctoral Fellowship. US Army Breast Cancer Research Program (DAMD17 - 97 -1- 7127)
1999	Young Investigator Award. The American Society of Gene Therapy
1998	Thomas Baum Award. The University of Michigan Department of Pharmacology
1996-1997	Student Research Award. The University of Michigan SPORE in Prostate Cancer
1995-1996	Student Research Award. The University of Michigan SPORE in Prostate Cancer
1995	The American Society of Hematologists Summer Research Fellowship
1994	The American Society of Hematologists Summer Research Fellowship
1993	Kent County Medical Society Distinguished Pre-Medical Student Award
1991-1993	Clarence & Ann Beets Memorial Scholarship in Biologic Sciences - Calvin College
1990-1991	Grand Rapids Foundation Award
1988-1993	Honors Program in Chemistry & Biochemistry – Calvin College
1988-1992	National Merit Scholarship

Professional Experience

2001	Postdoctoral Research Scientist. The Department of Radiation Oncology. The University of Michigan Health System. Mentor: Alnawaz Rehemtulla, Ph.D
2001	Tutor. Medical Physiology. The University of Michigan Medical School
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2000	Small Group Leader. Cell & Molecular Biology Seminars for first year medical students. The University of Michigan Medical School.
1995-1999	Graduate Student Research Assistant. Pharmacology. The University of Michigan
1997	Graduate Student Teaching Assistant. Pharmacology. The University of Michigan
1995	Research Rotation. Pharmacology. The University of Michigan Mentors: Alnawaz Rehemtulla, Ph.D & Jonathan Maybaum, Ph.D

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1992	Research Assistant. Biology. Calvin College.
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1990-1993	Teaching Assistant. Inorganic & Organic Chemistry. Calvin College

Volunteer Experience

2001	Soccer Coach. 2nd grade girls. Ann Arbor Recreation & Education
2000-present	Board Member. Ann Arbor Young Peoples Theater
1997-present	Volunteer. Inter-Faith Hospitality Network. Preparing meals for homeless families.
1998	Basketball coach. 4th grade boys. Ann Arbor Recreation & Education
1997-1998	Den Leader. Angell Elementary School Cub Scout Troop
1995-1997	Admissions Committee Member. The University of Michigan Medical School
1995-1997	MSTP Program Activities Committee. The University of Michigan Medical School
1994-1996	Member & Chairperson. The School Improvement Team - Angell Elementary School
1994	Soccer Coach. Kindergarten boys. Ann Arbor Recreation & Education
1993	Medical Volunteer at the Lynn Hadley Clinic for the homeless and uninsured.

Memberships

1998-present	The American Society of Gene Therapy (ASGT), Associate Member
1993-2001	American Medical Association - Medical Student Section (AMA-MSS), Member
1993-2001	American Medical Student Association (AMSA), Member

Peer Reviewed Publications

1. Blankespoor, R.L., De Jong, R.L., Dykstra, R., **Hamstra, D.A.**, Rozema, D.B., VanMeurs, D.P., Vink, P. (1991) Photochemistry of 1-Alkoxy- and 1-(Benzyloxy)-9,10 anthraquinones in Methanol: A delta-Hydrogen Atom Abstraction Process That Exhibits a Captodative Effect. *J. Amer. Chem. Soc.* 113: 3507-3513.

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9. **Hamstra, D.A.,** Pagé, M., Maybaum, J., and Rehemtulla, A. (2000) Expression of Endogenously Activated Secreted or Cell-Surface Carboxypeptidase A Sensitizes Tumor Cells to MTX-α-Peptide Prodrugs. *Cancer Res.* 60:657-665.

10. Chinnaiyan, A.M., Prasad, U., Shankar, S., **Hamstra, D.A.,** Shanaiah, M., Chenevert, T.L., Ross, B.D., and Rehemtulla, A. (2000) Combined effects of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci USA.* 97:1754-1759.

11. Stegman, L.D., Rehemtulla, A., **Hamstra, D.A.,** Rice, D.J., Jonas, S.J., Stout, K.L., Chenevert, T.L., and Ross, B.D. (2000) Diffusion MRI detects early events in the response of a glioma model to yeast cytosine deaminase gene therapy strategy. *Gene Therapy.* 7:1005-1010.

Abstracts & Presentations

1. **Hamstra, D.A.** and Rehemtulla, A. Oral Presentation & Poster Presentation. Endogenous activation of CPA mutants for cancer gene therapy. "Gordon Conference on Molecular Aspects of Radiation Oncology." June 29 - July 4, 1997. Plymouth State College, Plymouth, NH.

2. **Hamstra, D.A.** and Rehemtulla, A. Poster Presentation. Carboxypeptidase A (CPA) mutants and methotrexate-α-peptide prodrugs for virus dependent enzyme prodrug therapy (VDEPT). "Annual Meeting of the American Association of Cancer Research." March 28 - April 1, 1998. New Orleans, LA.

3. Nambu, Y., Hughs, S.J., Rehemtulla, A., **Hamstra, D.,** Orringer, M.B., and Beer, D.G. Poster Presentation. Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinoma. "Annual Meeting of the American Association of Cancer Research." March 28 - April 1, 1998. New Orleans, LA.

4. **Hamstra, D.A.** and Rehemtulla, A. Oral Presentation. Expression of Endogenously Activated Soluble or Cell-Surface Carboxypeptidase A Sensitizes Tumor Cells to Methotrexate Prodrugs. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

5. **Hamstra, D.A.**, Rice, D.J., Oyedijo, D., Ross, B.D., and Rehemtulla, A. Oral Presentation. Yeast Cytosine Deaminase is Functionally Superior to E. Coli Cytosine Deaminase in Enzyme Prodrug Cancer Gene Therapy *In Vitro* and *In Vivo*. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

6. Rice, D.J., **Hamstra, D.A.**, Pu, A., Oyedijo, D., Ross, B.D., and Rehemtulla, A. Poster Presentation. Bacterial Cytosine Deaminase Gene Therapy and Radiotherapy Inhibit Tumor Growth and Enhance Survival in an *In Vivo* Model of Head and Neck Cancer. "2nd Annual Meeting of the American Society of Gene Therapy." June 9 – June 13, 1999. Washington D.C.

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